COLLAGEN MEMBRANE DEGRADATION DELAYED BY TETRACYCLINE AND ITS SEMI-SYNTHETIC ANALOGUE DOXYCYCLINE - AN IN VITRO STUDY

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BRANCH – II
PERIODONTOLOGY

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY
Chennai – 600 032

2010 - 2013

CERTIFICATE

This is to certify that **Dr. GAURAV ARORA**, Post Graduate student (2010-2013) in the Department of Periodontics, Tamil Nadu Government Dental College and Hospital, Chennai - 600 003, has done this dissertation titled **"COLLAGEN MEMBRANE DEGRADATION DELAYED BY TETRACYCLINE & ITS SEMI-SYNTHETIC ANALOGUE DOXYCYCLINE – AN IN VITRO STUDY"** under our direct guidance and supervision in partial fulfillment of the regulations laid down by the **Tamil Nadu Dr.M.G.R. Medical University**, Chennai - 600 032 for **M.D.S.,** (Branch-II) **Periodontics** degree examination.

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DECLARATION

TITLE OF DISSERTATION	Collagen membrane degradation delayed by tetracycline & its semi-synthetic analogue doxycycline - An in vitro study
PLACE OF STUDY	Tamil Nadu Government Dental College & Hospital, Chennai-600003
DURATION OF THE COURSE	3 Years
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And

Mrs **Dr. Maheaswari Rajendran** aged 48 years working as **Professor** / Asst. Professor at the college, having residence address at No 9, old No 14, Muthupandiyan Avenue, Santhome, Chennai (hereinafter referred to as the 'PG/Research and Principal Investigator')

And

Mr. **Dr. Gaurav Arora** aged 28 years currently studying as **Post Graduate Student** in the Department of Periodontics herein after referred to as the 'PG/Research student and co- investigator').

Whereas the 'PG/Research student as part of her curriculum undertakes to research on **COLLAGEN MEMBRANE DEGRADATION DELAYED BY TETRACYCLINE & ITS SEMI-SYNTHETIC ANALOGUE DOXYCYCLINE - AN IN VITRO STUDY** for which purpose the PG/Principal investigator shall act as Principal investigator and the College shall provide the requisite infrastructure based on availability and also provide facility to the PG/Research student as to the extent possible as a Co-investigator

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In witness whereof the parties hereinabove mentioned have on this the day month and year herein above mentioned set their hands to this agreement in the presence of the following two witnesses.

College represented by its **Principal** PG Student

Witnesses Student Guide

1.

2.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
$Cacl_2$	Calcium chloride
CMT	Chemically modified tetracycline
COPD	Chronic obstructive Pulmonary disease
CRP	C Reactive Protein
Cu	Copper
ePTFE -	Poly tetrafluoroethylene
GCF	Gingival Crevicular fluid
Hg	Mercury
IL β	Interleukin beta
kDa	Kilodalton
LJP	Localized Aggressive Periodontitis
MMP	Matrix mettaloproteinases
Mm	mili molar
NaCl	Sodium chloride
PBS	Phosphate buffer solution
PGLA	Polylactic co- glycolic acid
PLA	Polylactic acid
PMN	Polymorphonuclear leukocyctes
Sn	Tin
TIMP	Tissue inhibitor of metalloproteinases
TNF α	Tumor necrosis factor alpha
TTC	Tetracycline
µl	microliters
Zn	Zinc

ABSTRACT

BACKGROUND:

Successful periodontal regeneration relies mainly on the re-formation of an epithelial seal, deposition of new acellular extrinsic fiber cementum and insertion of functionally oriented connective tissue fibers into the root surface, and restoration of alveolar bone height. The use of collagen membranes for treatment of periodontal defects in regenerative procedures are well known. Collagenase are enzymes responsible for degradation of collagen membranes which is detrimental to the success of periodontal regeneration. It has been well proven that tetracycline and doxycyline have role in inhibition of collagenase enzyme.

AIM OF THE STUDY:

To evaluate the effects of varying concentration of tetracycline and doxycycline on the rate of degradation of bioresorbable collagen membranes by clostridial collagenase when immersed in vitro.

MATERIALS AND METHODS:

The rate of collagen membrane degradation were analyzed quantitatively and qualitatively using spectrophotometric and microscopic analysis respectively in vitro when collagen membranes were incubated in PBS alone (group A), Tetracycline hydrochloride at varying concentration with PBS (group B) and Doxycycline hyclate at varying concentration with PBS (group C) observed over day 2, 4, 7 and 14 respectively . Groups were further divided into group I (treated with collagenase) and group II (treated without collagenase). Free protein content in the medium of each sample were analyzed using spectrophotometric analysis. Each collagen membrane sample was observed microscopically to get the overview of general surface topography and organization of collagen fibrils after incubation over time.

RESULTS:

There was a statistically significant difference in free protein release in the medium over the time between groups with and without collagenase and also there was a statistically significant difference between group with tetracycline and group without any drug and between group with doxycyline and group without any drug and between group with tetracycline and doxycycline in chlostridial collagenase.

CONCLUSION

Tetracycline at 50 mg/ml concentration and doxycycline at 20 mg/ml concentration are effective in delaying collagen membrane degradation in clostridial collagenase in vitro. Also doxycyline 20 mg/ml is more effective in prolonging the collagen membrane degradation time when compared to tetracycline 50mg/ml concentration.

Keywords: Tetracycline hydrochloride, doxycycline hyclate, collagen membrane degradation, collagenase

INTRODUCTION

The main goal of periodontal therapy is the predictable regeneration of the lost periodontium which allows repopulation of periodontal ligament cells into the wound area after periodontal surgery and also prevents the colonization of the microorganisms on the exposed root surface of the teeth. Successful periodontal regeneration mainly depends upon the re-formation of an epithelial seal, deposition of new acellular extrinsic cementum and insertion of functionally oriented connective tissue fibers into the root surface, and also the restoration of alveolar bone height[11].

Use of Collagen membranes for the treatment of periodontal defects in regenerative procedures are well known. Initially non resorbable barrier membranes such as ePTFE were mostly used for the purpose of bone regeneration and guided tissue regeneration procedures, but due to their several limitations, they were replaced by bio-resorbable membranes which are mainly composed of duramater, polyglycolic acid, polylactic acid, polyurethane and collagen. Bio absorbable membranes helps in maturation of newly formed tissues only if its structural integrity is preserved for sufficient period of time. Various materials have been used to delay the collagen membrane degradation.

Matrix metalloproteinases, zinc dependent enzymes are responsible for the degradation of collagen membranes which is detrimental to the success of periodontal regeneration. They are considered to be key initiators of collagen degradation, thus they are contributing to bone resorption in inflammatory diseases. Alteration in collagen degradation can be achieved by either cross linking which increases the

structural integrity or by delaying the degradation process of collagen. MMP inhibitors can modify the collagen degradation process.

Tetracycline have long been used in treatment of periodontal diseases due to their antimicrobial properties and their intrinsic anti-inflammatory activity. Tetracycline has inhibitory effects on matrix metalloproteinases which provides a therapeutic advantage in Periodontal regeneration[22]. Doxycycline, a semisynthetic tetracycline has been effective in reducing excessive collagenase activity in the gingival crevicular fluid of chronic periodontitis patients[25].

Hence this study has been undertaken to evaluate the effects of varying concentration of tetracycline hydrochloride and doxycycline hyclate on the rate of degradation of bioresorbable collagen membranes by clostridial collagenase when immersed in vitro.

AIM AND OBJECTIVES

Aim:

To evaluate the effects of varying concentration of tetracycline and doxycycline on the rate of degradation of bioresorbable collagen membranes by clostridial collagenase when immersed in vitro.

Objectives:

1. To evaluate and compare the effects of varying concentration of tetracycline with and without collagenase on the amount of protein released in the medium in vitro
2. To evaluate and compare the effects of varying concentration of doxycycline with and without collagenase on the amount of protein released in the medium in vitro
3. To visualize microscopically the degradation of collagen membrane in varying concentration of tetracycline, doxycycline and in absence of any drug respectively.
4. To compare the effects of tetracycline and doxycyline on the rate of degradation of collagen membrane in vitro.

REVIEW OF LITERATURE

COLLAGEN MEMBRANE DEGRADATION AND ENZYME INVOLVED

The use of Collagen membranes for the treatment of Periodontal defects in regenerative procedures such as Guided tissue regeneration is widespread. Initially non resorbable barrier membranes were used but later on Bio resorbable membranes were introduced to overcome the limitations of non resorbable membranes.

As compared with non-resorbable membranes, resorbable collagen membranes show a lower incidence of spontaneous exposure to the oral environment, and also unlike non-resorbable membranes, infection is not associated with soft tissue healing following exposure of resorbable collagen membranes. (**Friedmann et al 2002**)[13]. Collagenase enzyme is known to be responsible for the degradation of collagen membranes which is detrimental to the success of regenerative procedures.

Two possible mechanisms of collagenolysis **(Weiss JB 1976)**[82] in vivo:

a) Collagenase, an enzyme is capable of initiating collagen degradation by cleaving a single peptide bond on the helical portion of the three subunit chains of the native substrate and continuing the further breakdown of the

reaction products unaided by other enzymes but helped by their denaturation at physiologic temperature

b) the multiple enzyme mechanism suggests that several enzymatic steps are necessary to degrade native insoluble collagen, starting with depolymerization (catalyzed by nonspecific proteolytic enzymes), followed by specific cleavage by the collagenases, and continued by digestion of denatured reaction products, first by specific endopeptidases and exopeptidases, then by peptidases of low specificity, and finally by nonspecific exopeptidases.

The rate-limiting factor in physiologic collagen degradation are variations in enzyme activity and concentration and changes in substrate susceptibility.

In 1953, MacLennan et al[43]**,** showed the beginning of study on collagenases with recognition of the involvement of the proteolytic clostridia in tissue putrefaction and the subsequent isolation of clostridium histolyticum, an extracellular enzyme that was able to digest tendons.

Gibbons, R. J., and J. B. MacDonald[16] in **1961** reported first organism possessing collagenolytic activity in the human oral cavity.

In 1962, Gross and Lapie're[29] described an in vitro method where they demonstrated the existence of endogenous specific collagenase in animal tissues. They also reasoned that an enzyme such as collagenase was a potentially dangerous substance, so that a regulation of synthesis, activity, and diffusion was essential

Rippon, J. W[62] in **1968**, reported in a case of human mycetoma, that isolated strain of Actinomadura (Streptomyces) madurae was shown to produce a collagenase with activity against native collagen. **Kivirikko KI et al**[38] in **1970** established that a small but a significant fraction of total body collagen is rapidly synthesized and degraded throughout life, and this fraction is quantitatively different in various tissues, and that abnormal collagen degradation, either by excess or deficiency, might play a role in some human diseases.

Fullmer HM 1971, **Page RC, Schroeder HE**[14] in **1973**, suggested that increase in collagenase production has been found in situations in which massive collagen degradation is clearly documented, e.g. periodontal disease.

Ohlsson K, Olsson I[55] in **1973**, reported about specificity of collagenases for collagen, the enzymes seem to attack few or no other proteins; the exception to this is leukocyte collagenase, which in highly purified form will also degrade fibrinogen and proteoglvcans. Then in **1973, Harris ED Jr. Krane SM**[32]**,** also found that kinetics of collagenolytic attack depends upon the degree of aggregation and cross linking of the substrate. Under identical conditions of incubation, the soluble collagen molecules are more susceptible than reconstituted fibrils and the susceptibility decreases with the number of cross-links in the substrate.

In 1974, Robertson et al[63], suggested that bacterial proteinases may be capable of activating latent mammalian collagenases, thus they contribute to the degradation of collagen indirectly. **Montfort I, Perez-Tamayo R**[48] in **1975,** surveyed many different tissues of the normal rat and then detected collagenase bound to the three major histologic types of collagen, i.e. collagen

bundles, reticulum fibers, and basement membranes and finally stated that collagenases are present in the extracellular structures of normal animals.

Weiss JB[82] in **1976** established that pure preparations of almost all types of animal collagenases cleave the native soluble collagen or reconstituted collagen fibrils at a single peptide bond, when incubation is carried out at physiologic pH and ionic strength and at temperatures which is below the denaturation point of the reaction products (30 C).

Steven FS[75] in **1976** then found in his study that when a 0.02% solution of human skin collagenase was incubated with approximately 2 to 3 mg of whole fresh human skin dermis at 37 C for 14 hours, approximately 25% of the hydroxyproline in the tissue is released into the medium, when compared with no liberation of hydroxyproline in the absence of the enzyme.

In 1977, Stricklin GP et al[76] stated that collagenases have been claimed to occur in animal tissues and fluids in at least in three molecular forms: a) latent enzyme, b) free enzyme, and c) collagen-bound enzyme.; **Stricklin et al** also claim that these molecular forms of collagenase are true zymogens or precursors of the active form of the enzyme, which are synthesized and secreted by different cells as such. Such proenzymes would require their activation by specific mechanisms, which may be partial proteolysis by other enzymes, so-called auto-activation

Horwitz AL. Hance AJ. Crystal RG[34] in **1977,** suggested that the influence of the genetic type of collagen on its susceptibility to specific collagenase attack is the difference in the rate of degradation of Types I and III by polymorphonuclear leukocyte collagenase

In 1979, Smith, L. D. S.[69] found that clostridial collagenases were the first enzymes of this group to be identified and characterized and have become the benchmark against which newly discovered collagenolytic enzymes are later compared. *Clostridium perfringens* is the most common pathogen of clostridial myonecrosis (gas gangrene) and has also been identified as a major etiological agent in other necrotizing diseases, including enteritis necroticans, necrotizing enteropathy, necrotizing pneumonia, and gangrenous cholecystitis. The organism has been shown to produce a large number of tissue-destroying enzymes including collagenase which is known as kappa toxin.

Then in 1984 Bond, M. D., and E. Van Wart[7]**,** reported a second clostridial species associated with myonecrosis, *C. histolyticum*, which has capability to produce upto six electrophoretically different collagenases

Sorsa T et al[70] in **1988**, suggested that PMNs are the cells that provide the major source of collagenase that mediates tissue breakdown during inflammatory periodontal disease, whereas fibroblasts contribute the collagenase required for connective tissue remodeling in normal gingiva.

Jin, K.et al[36] in **1989**, suggested that Porphyromonas gingivalis is the only black-pigmented anaerobic rod that produces significant collagenase activity under the appropriate assay conditions. **Harrington, D. J., and R. B. Russell**[31] in **1994**, suggested that these enzymes may contribute to the breakdown of the collagen component of both dentine and cementum in the pathogenesis of dentinal and root surface caries.

Lekovic V et al[42] in **1997**, stated that there is no need for a second surgical intervention for removal of the bio-absorbable membrane along which they present improved soft tissue healing, the incorporation of the collagen

membranes by the host tissues and rapid resorption if exposed eliminate open microstructures prone to bacterial contamination

Lee SJ et al[41] in **2001**, stated that barrier membranes must satisfy space maintenance, tissue integration, cell-occlusiveness, and biocompatibility criteria. Among these properties, optimal space maintenance is the most important property to ensure the success of the periodontal treatment. Space maintenance by the barrier membrane is necessary to withstand the forces exerted by the overlying soft tissue flaps, to prevent the collapse of the soft tissue, and also to maintain wound space

In 2003, Michael N. Sela et al[47] examined the role of periodontal bacteria and their enzymes in the degradation of commercially used collagen membranes. Degradation of two collagen membranes (Biomend and Bio-Guide) labelled by fluorescein isothiocyanate was examined by measuring soluble fluorescence. Porphyromonas gingivalis, Treponema denticola and Actinobacillus actinomycetemcomitans and their enzymes were evaluated. Collagenase from Clostridium histolyticum was used as a positive control. These results suggested that proteolytic bacterial enzymes may take part in the degradation of barrier membranes used for the guided tissue regeneration.

Moses et al[49] in **2005**, found in his comparative study between the prematurely exposed non-resorbable membranes (ePTFE), non-cross-linked collagen membranes (BioGide) and cross-linked collagen membranes (Ossix), the latter were claimed to be superior, and capable of supporting healing.

Schwarz et al[65] in **2006,** proposed that the vascularization process may also be contributed to membrane degradation since the monocytes penetrating through the blood vessel wall may differentiate into macrophages

Ofer Moses et al[53] in **2010**, found in his study that immersion of collagen membranes in TTC solution prior to their implantation and systemic administration of TTC significantly decreased the membranes' degradation in vivo

ROLE OF MATRIX-METALLOPROTEINASES IN PERIODONTAL INFLAMMATION

Matrix metalloproteinases are the zinc dependent enzymes produced both by infiltrating and resident cells of the periodontium, play a role in

(i) physiological (such as tooth eruption) and

(ii) pathological (such as periodontitis) events.

An imbalance between activated matrix metalloproteinases and their host-derived endogenous inhibitors leads to the pathological breakdown of the extracellular matrix during periodontitis and other diseases (**Birkedal-Hansen H 1993)**[5]

In 1962, Gross J, Lapiere C[29] reported first the prototype of host-derived, connective tissue destructive matrix metalloproteinases, namely interstitial collagenase (matrix metalloproteinase-18 or *Xenopus* collagenase-483). They also found an active enzyme in the culture media of tissue

fragments of the tail fin skin that degraded the triple helix of native type I collagen.

Golub L et al[18] in **1985** reported the detection of the elevated levels of active rather than latent collagenase in the fluid of the periodontal pocket and in the extracts of the adjacent inflamed gingival tissue.

Sorsa T et al[70] in **1988** stated that the influence on the source of collagenolytic activity may arise from the level of extracellular activation, as evidence exists that polymorphonuclear leukocyte–type and fibroblast-type matrix metalloproteinases may respond differently, in the extracellular matrix, to factors that activate their respective zymogens

Birkedal-Hansen H[5] **1993**, found that during inflammatory destruction, the most important component of lost periodontium is the collagen type I that is found in the periodontal ligament and the alveolar bone organic matrix. Four distinct pathways may be involved with this destruction: (i) Plasminogen-dependent, (ii) phagocytic, (iii) osteoclastic, and (iv) MMP pathway. They also reported that Gingival fibroblasts, keratinocytes, resident macrophages and polymorphonuclear leukocytes (PMN) are capable of expressing MMP-1, MMP-2, MMP-3, MMP-8, MMP-9. Later on in 1993 again, they reported that matrix metalloproteinases are expressed in response to specific stimuli by resident connective tissue cells as well as the major inflammatory cell types that invade the tissue during remodeling events in vivo

Makela M et al[44] in **1994** reported that MMPs are present in both the active and latent forms in chronically inflamed gingival tissues and gingival crevicular fluid. Active collagenase and gelatinase were found in the

crevicular gingival fluid of patients with periodontitis in much larger amounts than in control subjects

Golub L et al[23] in **1995** reported that Inflammatory cells such as neutrophils and macrophages produce matrix metalloproteinases, with neutrophils being the major source of collagenase and gelatinase in inflammatory diseases such as periodontitis. They also reported that matrix metalloproteinase-8 was found to be the main interstitial collagenase in gingival extracts and gingival crevicular fluid

Van der Zee E[79] in **1996** stated that MMPs are the major players in collagen breakdown during periodontal tissue destruction

Ramamurthy MS et al[61] in **1998** reported that the topical application of CMT-2, an inhibitor of MMPs activity, can enhance wound healing in diabetic rats. They also reported that the treatment with non-antimicrobial tetracycline prevents not only the destruction of periodontium by MMPs, but also avoids the exposure of roots to host tissue.

Opdenakker G et al[56] **1998**, suggested that during an inflammatory response, leukocyte trafficking through tissue barriers, including basement membranes, is only possible if these cells are equipped with enzymes that can remodel the extracellular matrix.

Souza AP et al[73] **2000,** demonstrated that divalent metal salts, as Zn, Cu, Hg and Sn, are capable to inhibit the activity of MMP-2 and MMP-9 at low concentration. **Gerlach RF et al**[15] **2000**, stated that lead, cadmium and zinc inhibit the activity of enamel matrix proteinases in vitro. **Kleifeld O et al**[39] **2000**, reported that when cells produce MMPs, most of the enzymes are secreted in a latent pro-form and removal of the pro-peptide (about 10 kDa)

from the active site, for example, by proteolysis, leads to activation of the enzymes

Uchida M et al[78] **2001**, demonstrated that MMP3, MMP9, and MMP13 mRNA levels are increased when osteoblast cultures are stimulated by resorptive factors such as cytokines interleukin (IL)-1b and tumor necrosis factor (TNF)-α, parathyroid hormone, and prostaglandin E_2 .

Visse R et al[80] **2003** suggested that in addition to regulation by activation processes and gene expression, the activities of MMPs are also controlled by the four natural tissue inhibitors of metalloproteinases (TIMPs).

Bildt et al[4]**.** in **2008** reported that the total MMP-9 levels and its active form have been demonstrated to significantly increase with periodontal inflammation in comparison to controls, composed of gingivitis and healthy subjects, and to drop along with inflammation after periodontal therapy

Kessenbrock et al[37] **2010** stated that MMPs share a basic structure composed of three domains, namely the pro-peptide, catalytic and the hemopexin-like domain; the latter is linked to the catalytic domain via a flexible hinge region. The proteolytic activity of MMPs is subjected to a complex regulation that involves three major steps 1) gene expression, 2) conversion of zymogen to active enzyme and 3) specific inhibitors.

Sorsa et al[72] **2011, Buduneli et al**[8] **2011**found that the patho-physiological significance of increased MMP expression in periodontitis will rely ultimately on the presence of endogenous inhibitors and activating enzymes that will determine overall MMP activity

INHIBITION OF MATRIXMETTALOPROTEINASES BY TETRACYCLINE

Periodontal pathogenic bacteria such as Treponema denticola and P. gingivalis, were

shown to adhere to different barrier membranes in vitro, and cause rapid degradation

of collagen membranes. Since early degradation of collagen membranes is detrimental to the success of regenerative procedures, various materials include tetracycline impregnation, doxycycline, chlorhexidine dihydrochloride which delayed collagen membrane degradation, has inhibitory effects on bacterial adherence and early degradation is of great importance in periodontics as membrane loaded with these materials might have enhanced regenerative efficacy.

In 1954, Stephens CR et al[74] reported the first chemically purified tetracycline, chlortetracycline. Tetracyclines were discovered in 1948 as natural fermentation products of a soil bacterium, Streptomyces aureofaciens.

Gordon et al[25] **1981**, stated that tetracyclines have a unique ability, antibiotics to concentrate in the GCF of the periodontal pocket at levels 5-10 times greater than those found in serum.

Slots J[68] **1983**, reported that tetracycline has been shown to be considerable benefit in the treatment of aggressive periodontitis (AP) in which the prime pathogen, Aggregatibacter actinomycetemcomitans, is very susceptible to the antibiotic

Goodson JM et al[24] **1983**, reported that tetracyclines are usually given orally, although topical application have been used in periodontal treatment regimen.

Golub et al[17] **1984**, reported that non-anti-microbial properties of chemically modified tetracyclines (CMTs) show great promise in their therapeutic value. These properties consist of the inhibition of mammalian collagenase. Then in **1985**, they found that tetracycline, doxycycline, and minocycline can all suppress the activity of the tissue enzyme collagenase as determined by its presence in crevicular fluid. In **1990**, they stated that the ability of pharmacologic concentrations of tetracyclines to inhibit PMN but not fibroblast collagenase may be therapeutically important; therapy with these drugs would be expected to reduce pathologically elevated collagenolytic activity (e.g., during inflammation), but not the collagen turnover required to maintain normal tissue integrity.

Chopra et al[10] **1985** stated that tetracyclines are bacteriostatic inhibitors of protein synthesis. They accumulate intracellularly by way of energy dependent transport systems present in bacterial membrane

O'Connor BC et al 1990[52], reported that strict anaerobic bacteria are susceptible to tetracyclines, although some black-pigmented bacteroides have been reported to be minocycline-resistant

Golub et al[19] **1991**, stated that in addition to interstitial collagenase, other matrix metalloproteinases (MMPs) inhibited by tetracycline include type IV collagenase/gelatinase, stromelysin, and macrophage elastase. The anti-collagenolytic activity of tetracycline is independent of its antibacterial property

Parashis and Mitsis[57] in **1992** evaluated the potential effect of tetracycline root preparation on regeneration in Class II furcation defects found no additional improvement in the sites treated with guided tissue regeneration in conjunction with tetracycline as compared with barrier membrane placement alone.

Grevstad HJ et al[27] **1993** reported that tetracycline besides having antimicrobial activity are also able to inhibit the activity of interstitial collagenases present in variety of cells such as neutrophils and macrophages. **Ingman T et al**[35] **1993**, reported that Human PMNs are the source of the crevicular fluid collagenase that is most susceptible to tetracyclines, while that from fibroblasts, which is the source of collagenase in LJP, is relatively resistant

Golub et al[21] **1994**, found that main therapeutic mechanism of action of tetracycline is a direct block of already-active collagenase in the periodontal pocket or inhibition of the activation of latent forms of collagenase. They also reported that tetracyclines have been shown to block the proenzyme conversion to an active state, to block the active enzyme, and to inhibit reactive oxygen species that may be involved in activation of MMPs and also found in his study that inhibition of MMPs by tetracyclines is unrelated to the antibacterial action.

Shapira LL et al[66] **1996**, reported that tetracyclines, besides acting as antibiotics, may also affect inflammation, immunomodulation, cell proliferation, and angiogenesis.

Chung et al[12] **1997**, stated that inflammatory cytokines including TNF-alpha, IL-1 beta, and IL-6 are markedly down-regulated in patients

during treatment with tetracyclines. This phenomenon also reduces the amount of MMP's present in inflamed tissues, contributing to a reduction of the collagenolytic activity

Ofer Moses et al[54] in **2001** proved that collagen membranes immersed in 50 mg/ml tetracycline hydrochloride solution exhibited the longest degradation time, both in clostridial collagenase and human bone lineage cell assays In vitro.

Acharya MR et al[1] in **2004**, reported that tetracyclines are antibiotics that also inhibit the breakdown of connective tissue. Chemically modified tetracyclines (CMTs) without antibiotic activities have several potential advantages over conventional tetracyclines due to the absence of gastrointestinal side effects or toxicity and higher plasma concentrations can be reached for prolonged periods of time.

Sang-Bae Lee et al[64] **2008**, found in his study that the collagen membrane has an antibacterial performance by the incorporation of tetracycline into a biodegradable polymer. In order to control the release rate of tetracycline, concentration of three polymers that have different molecular weight was changed and then it was coated on the collagen membrane. The antibacterial effect of PLA and PLGA was decreased when concentration of polymer was increased. Such result supports the result of drug release. Initial drug release was largely shown after 1 day. It seemed to largely increase antibacterial by releasing a large amount of drug when concentration of polymer was decreased.

Ofer Moses et al[53] **2010**, found in his study that immersion of collagen membranes in TTC solution prior to their implantation and systemic

administration of TTC significantly decreased the membranes' degradation in vivo

INHIBITION OF MATRIXMETTALOPROTEINASES BY DOXYCYCLINE

Walker CB et al[81] **1985**, found that locally used doxycycline has been shown to concentrate in crevicular fluid, successfully eliminated Actinobacillus actinomycetemcomitans and demonstrated a wide spectrum of activity against other suspected periodontal pathogens

In 1986, Mandell RL et al[46] suggested that a combination of surgical debridement plus systemic doxycycline for 14 days was effective in eliminating or suppressing A.a.comitans from periodontal pockets, but this emphasized the possibility of re-infection from other oral sites or incomplete elimination.

Pascale D et al[58] **1986**, found that doxycycline achieved gingival fluid levels of 4 to 10 Vg/mol after the administration of 100 mg every 12 hours for the first day, followed by 100 mg/day for 14 days.

Golub et al[20] **1990**, reported that patients with adult periodontitis were administered either 30 mg doxycycline BID or a placebo for two weeks. Patients received oral hygiene instructions, scaling and root planing, and surgery which included the removal of gingiva and the collection of Gingival Crevicular fluid. A reduction in extracellular collagenase activity by approximately 60-80% was seen in the crevicular fluid of periodontal pockets and in the gingival tissue

Kulkarni GV et al[40] **1991** reported that in patients identified as having refractory periodontitis, based on recent attachment loss > 2 mm and the presence of periodontal abscesses despite the regular periodontal supportive treatment, administration of doxycycline for three weeks showed no further disease activity for up to seven months. Most pathogens were reduced except for A.a.comitans. This long-term effect may be due to a combination of doxycycline's antibacterial and anti-collagenolytic action.

Grevstad HJ & Boe OE[28] **1995** found that doxycycline, the most potent and cost effective tetracycline commercially available, has been shown to reduce the collagenase production by osteoblasts and osteoclasts and also to delay osteoclasts recruitment following dental surgery.

Ashley RA et al[2] **1999** found that the therapeutic action of doxycycline witnessed is primarily due to the modulation of the host response because the low-dose formulations of these drugs have lost their antimicrobial activity. **Thomas J, Walker C & Bradshaw M**[77] **2000**suggested that low dose doxycycline decreases attachment loss and excessive collagenase activity in Crevicular fluid of periodontitis patients

Sorsa T et al[71] **2006** reported that the tetracycline analogue, doxycycline hyclate, available for use specifically in periodontal disease, is the only collagenase inhibitor approved by the United States Food and Drug Administration (FDA) for any human disease.

Prashant S. Dalvi et al[59] **2011** found the beneficial effect of anti-inflammatory and MMP-inhibiting property of short-term doxycycline in lung function parameters and systemic inflammatory marker, CRP in patients of stable COPD.

MATERIALS AND METHODS

3.1 Materials:

Preparation of Collagen membrane

Collagen bio-absorbable membrane (periocol)

Digital weighing machine

Sterile cutting instrument

Tweezer

Preparation of Collagenase enzyme and Enzyme Buffer

Collagenase enzyme (Sigma Aldrich laboratories)

Collagenase enzyme buffer (500 ml)

- 50 mM Tricine (weight 4.47 g)
- 10 mM $Cacl_2$ (weight 0.55g)
- 400 Mm NaCl (weight 11.6 g)
- pH = 7.5

Digital pH meter

Preparation of Tetracycline Hcl and Doxycycline solution

Pure Tetracycline hydrochloride (500 mg capsules) (Sigma Aldrich laboratories)

Pure Doxycycline hyclate (100 mg capsules) (Sigma Aldrich laboratories)

Phosphate buffer saline

Test tubes

Test tube holder

Distilled water

Glass beaker

Magnetic stirrer

Digital pH meter

Normal Saline

Spectrophotometric analysis

Spectrophotometer

Bradford reagent

Spectrophotometer test tube (cuvettes)

Optical microscopic Examination

Optical Light microscope

Microscope slides

Cover slips

3.2 Methods:

3.2.1 Preparation of Collagen membrane

Collagen bio-resorbable membranes were cut into rectangular sheets of 5 X 10 mm (average measured weight of 5 ± 1 mg).

3.2.2 Preparation of Collagenase

3.2.2.1 Preparation of Collagenase enzyme buffer (500 ml)

Collagenase enzyme buffer were prepared from 50 mM Tricine (weight 4.47 g), 10 mM $Cacl_2$ (weight 0.55g), 400 Mm NaCl (weight 11.6 g) at pH = 7.5

3.2.2.2 Preparation of Clostridial Collagenase (15 collagen digestion units)

1 mg of collagenase contains 800 collagen digestion units

3 mg of collagenase mixed in 1 ml collagenase enzyme buffer contains 2400 collagen digestion units

1 µl of collagenase + collagenase buffer contains 2.4 collagen digestion units

6.25 µl collagenase + collagenase buffer contains 15 collagen digestion units

3.2.3 Preparation of Phosphate buffer solution

Phosphate buffer solution were prepared with 0.1 M concentration at pH= 7.4.

3.2.4 Incorporation of drugs to collagen membrane

3.2.4.1 Tetracycline hydrochloride

Four concentration of TTC-Hcl was prepared i.e. 5mg/ml, 20mg/ml, 50mg/ml, 100mg/ml

5mg/ml – 50mg TTC- Hcl powder in 10 ml saline

20 mg/ml – 200mg TTC- Hcl powder in 10 ml saline

50 mg/ml – 500mg TTC- Hcl powder in 10 ml saline

100 mg/ml – 1000mg TTC- Hcl powder in 10 ml saline

3.2.4.2 Doxycycline hyclate

Four concentration of Doxycycline hyclate was prepared i.e. 5mg/ml, 20mg/ml, 50 mg/ml, 100 mg/ml

5mg/ml – 50mg Doxycycline hyclate powder in 10 ml saline

20 mg/ml – 200mg Doxycycline hyclate powder in 10 ml saline

50 mg/ml – 500mg Doxycycline hyclate powder in 10 ml saline

100 mg/ml – 1000mg Doxycycline hyclate powder in 10 ml saline

Sample Groups

Group A (control) – Phosphate buffered solution alone

Group B (Test 1) – Tetracycline hydrochloride in Phosphate buffered solution

Group C (Test 2) – Doxycycline hyclate in Phosphate buffered solution

All the three groups were further divided into Group I & Group II

Group I – treated with collagenase

Group II – treated without collagenase

3.2.5 Incubation of Collagen membrane in PBS & Drugs

Incubation of Collagen membrane sheets were done in group A (control group - PBS alone) at 4^0C temperature for 24 hours

Incubation of Collagen membrane sheets were also done in four varying concentrations (5mg/ml, 20 mg/ml, 50 mg/ml, 100 mg/ml) of group B (Tetracycline in PBS) and group C (Doxycycline in PBS) respectively at 4^0C temperature for 24 hours.

3.2.6 Incubation of Collagen membrane in Collagenase

After 24 hours of incubation of collagen membrane in group A as well as group B and group C, 8the collagen membrane sheets were then rinsed with distilled water followed by incubation of collagen membrane sheets in Bacterial (Clostridial) Collagenase with collagenase enzyme buffer containing 15 collagen digestion units.

3.2.7 Spectrophotometric analysis

A series of protein standards using BSA diluted with 0.15 M NaCl to final concentrations of 0 (blank = NaCl only), 250, 500, 750 and 1500 µg BSA/Ml were

prepared. Also serial dilutions of the control group, group A and group B samples to be measured were prepared.

Then, 100 µL of each of the above samples were added to a separate test tube followed by addition of 5.0 mL of Coomassie Blue to each tube and mix by vortex, or inversion.

The spectrophotometer to a wavelength of 595 nm were adjusted, and blank using the tube which contains 0 BSA.

After 5 minutes, each of the standards and each of the samples at 595 nm wavelength were read.

The absorbance of the standards vs. their concentration were plotted. The extinction coefficient were computed and the concentrations of the unknown samples were calculated on day 2, 4, 7, 14. The data collected were statistically analyzed.

3.2.8 Optical Light Microscopic Examination

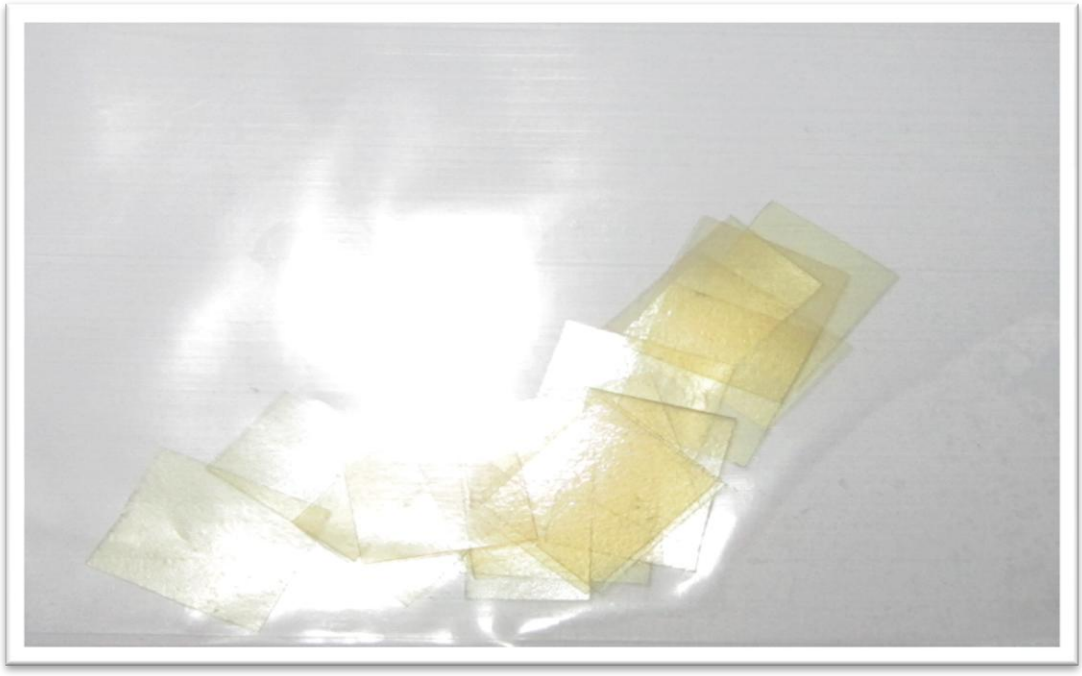
Collagen membrane sheet from each solution were observed on 2, 4, 7 and 14 days.

Samples were placed on a microscope slide and then covered with a coverslip and viewed under the microscope objective at magnification values of 40 X & 100 X.

Each collagen membrane samples were observed to get the overview of general surface topography and organization of collagen fibrils after incubation in group A I, B I, CI over the day 2, 4, 7 and 14.

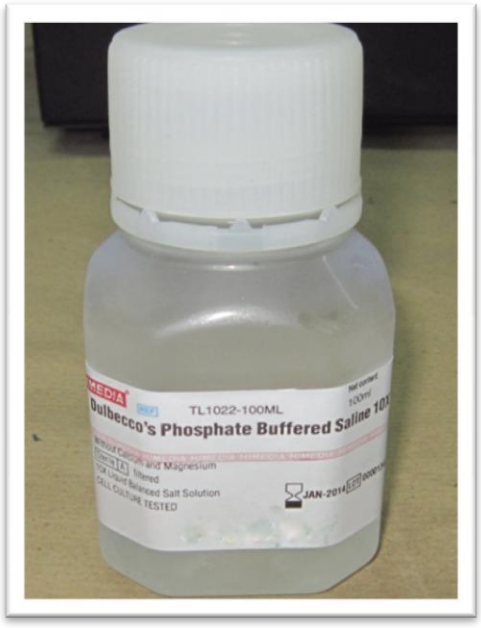
In order to avoid bias, the assessment of the morphological characteristics were done by a single investigator who was unaware of the origin of the specimen.

PHOTOGRAPH 1: ARMAMENTARIUM

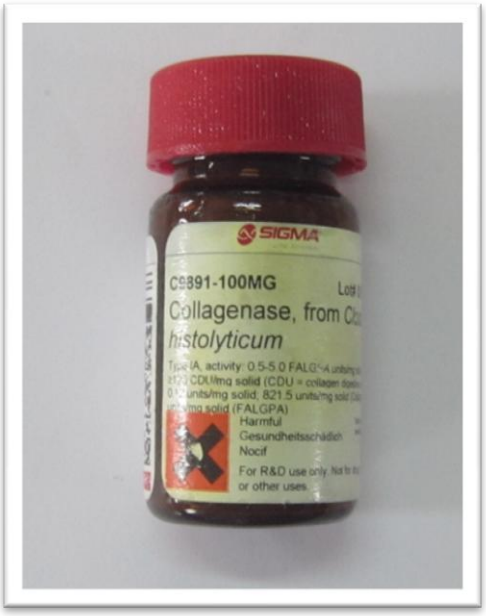


PHOTOGRAPH 2: BIORESORBABLE COLLAGEN MEMBRANES

REAGENTS USED:



PHOTOGRAPH 3:
PHOSPHATE BUFFER SOLUTION



PHOTOGRAPH 4:
CLOSTRIDIAL COLLAGENASE

PHOTOGRAPH 5:
BRADFORD REAGENT

PHOTOGRAPH 6:
COLLAGENASE BUFFER REAGENTS

PHOTOGRAPH 7:
TETRACYCLINE HYDROCHLORIDE CAPSULES

PHOTOGRAPH 8:
DOXYCYCLINE HYCLATE CAPSULES

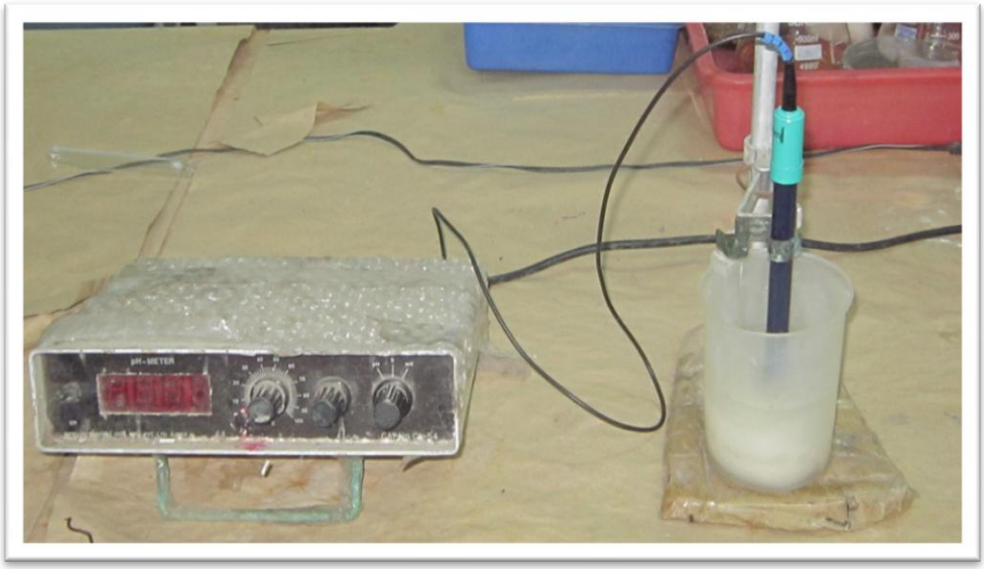
PHOTOGRAPH 9:
TTC & DOXYCYCLINE SOLUTION

PHOTOGRAPH 10:
SOLUTIONS - GROUP A, B, C

PHOTOGRAPH 11: SPECTROPHOTOMETER WITH CUVETTES

PHOTOGRAPH 12: OPTICAL LIGHT MICROSCOPE

PHOTOGRAPH 13: MAGNETIC STIRRER



PHOTOGRAPH 14: pH METER

PHOTOGRAPH 15: DIGITAL WEIGHTING MACHINE

PHOTOGRAPH 16: MICROSCOPIC SLIDE WITH COLLAGEN MEMBRANE TO OBSERVE UNDER MICROSCOPE

STATISTICAL ANALYSIS

The statistical package SPSS V 17 (Stastistical Package for social Science, version 17) was used for statistical analysis.

Student's Independent t-test

The independent t-test was used to compare the statistical significance of a possible difference between the means of two groups on some independent variable and the two groups were **independent** of one another. Here, independent t-test was used to compare the means values of free protein in medium between groups with and without collagenase.

The formula for the independent t-test was

$$t = \frac{X_1 - X_2}{\sqrt{\left(\frac{SS_1 + SS_2}{n_1 + n_2 - 2}\right)\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}},$$

where

X_1 is the mean for group 1,

X_2 is the mean for group 2,

SS_1 is the sum of squares for group 1,

SS_2 is the sum of squares for group 2,

$\mathbf{n_1}$ is the number of samples in group 1, and

$\mathbf{n_2}$ is the number of samples in group 2.

The t-value found was the difference between the two means divided by their sum of squares and taking the degrees of freedom into consideration.

$$SS_1 = \sum X_1^2 - \frac{\left(\sum X_1\right)^2}{n_1}$$

and

$$SS_2 = \sum X_2^2 - \frac{\left(\sum X_2\right)^2}{n_2}$$

The degrees of freedom for the independent t-test used was:

$$df = n_1 + n_2 - 2$$

One-way Analysis of Variance:

The ANOVA is used with one categorical independent variable and one continuous variable. The independent variable can consist of any number of groups (levels).

The formula for one way Analysis of Variance is

$$SS_{total} = \left(\sum x_1^{\,2} + \sum x_2^{\,2} + \dots \sum x_r^{\,2}\right) - \frac{\left(\sum x_1 + \sum x_2 + \dots \sum x_r\right)}{N}$$

$$SS_{total} = \left[\frac{\left(\sum x_1\right)^2}{n_1} + \frac{\left(\sum x_2\right)^2}{n_2} + \dots \frac{\left(\sum x_r\right)^2}{n_r}\right] - \frac{\left(\sum x_1 + \sum x_2 + \dots \sum x_r\right)}{N}$$

$$SS_{within} = SS_{total} - SS_{among}$$

$$df_{among} = r-1 \qquad df_{within} = N-r$$

$$MS_{among} = \frac{SS_{among}}{df_{among}} \qquad MS_{within} = \frac{SS_{within}}{df_{within}} \qquad F = \frac{MS_{among}}{MS_{within}}$$

x = individual observation

r = number of groups

N = total number of observations (all groups)

n = number of observations in group

Tukey's post-hoc test:

To answer the pair comparisons, we run a series of Tukey's post-hoc tests, which are like a series of t-tests.

$$\frac{M_1 - M_2}{\sqrt{MS_w\left(\frac{1}{n}\right)}}$$

M = treatment/group mean

n = number per treatment/group

The **P value** or calculated probability is the estimated probability of rejecting the null hypothesis (H0) of a study question when that hypothesis is true. The smaller the p-value, the more significant the result is said to be. All *P*-values are two tailed, and confidence intervals were calculated at the 95% level. Differences between the two populations were considered significant when $p < 0.05$.

RESULTS

In this study, 108 bioabsorbable collagen membrane samples were obtained and the samples were divided into three groups where each group was treated with 6 samples individually:

Group A (control group) was treated with Phosphate Buffer solution without any drug

Group B was treated with Tetracycline Hcl dissolved in Phosphate buffer solution each at 4 different concentrations of 5 mg/ml, 20 mg/ml, 50 mg/ml, 100 mg/ml

Group C was treated with Doxycycline hyclate dissolved in Phosphate buffer solution at 4 different concentrations of 5 mg/ml, 20 mg/ml, 50 mg/ml,100 mg/ml.

All the groups were further divided into two groups I, II based on treatment with collagenase enzyme where

Group I – treated with collagenase

Group II – treated without collagenase

These samples were observed for changes of free protein content in the medium using spectrophotometer at optical density @ 595 nm at 4 different incubation periods i.e. 2, 4, 7 and 14 days. These samples were also observed in the optical light microscope for the changes in surface topography of collagen membrane degradation and structural organization of collagen fibers on 2, 4, 7 and 14 days.

Table 1,2,3,4,5,6 shows the master chart for free protein content in Group A,B, C treated with collagenase enzyme (Group I) and Group A,B, C treated without

collagenase enzyme (Group II) consists of different values of protein content in medium on Day 2, 4, 7 & 14.

Table 7, 8 & Figure 1 shows mean values of free protein content in Group A treated with and without collagenase respectively on Day 2, 4, 7 & 14. Results shows that Group A treated with collagenase has more protein release than Group A treated without collagenase and new protein release increases till Day 7 and then start decreasing upto Day 14 for Group A treated with collagenase.

Table 9, 10 & Figure 2 shows mean values of free protein content of each of four different concentrations respectively in Group B treated with collagenase on Day 2, 4, 7 & 14. Results shows at 5 mg/ml concentration of Group B I, new protein release increases steadily till day 14 while at 20 mg/ml, it decreases at day 4 and then increased till day 14. At 50 mg/ml, it was decreased at day 4 and then again increases and finally decreased to lowest new protein release at Day 14 compared to other three concentrations including 100 mg/ml which has maximum protein release at day 14 although less protein release on Day 2 and 4 whereas mean values of free protein content of each of four different concentrations respectively in Group B treated without collagenase (B II) on Day 2, 4, 7 & 14 shows that at 5 mg/ml concentration, less new protein release in the beginning which increased till day 4 and again decreased to day 7 but the levels were more at Day 14. At 20 mg/ml, new protein release increased steadily upto day 7 and then decreased at day 14. At 50mg/ml, there was not much difference in new protein release at different days while at 100 mg/ml, the new protein release increased steadily upto day 14.

From the mean values it was found that after Day 2 incubation, collagen membrane immersed in Group B I at 5 mg/ml solution exhibited 45 % less protein release into

the medium (less membrane degradation) while which immersed in 20mg/ml, 50 mg/ml and 100 mg/ml exhibited 50%, 30% and 39% less membrane degradation respectively when compared with group A I. On day 4, Group B I at 5 mg/ml solution exhibited 38 % less protein release into the medium while which immersed in 20mg/ml, 50 mg/ml and 100 mg/ml exhibited 60%, 55% and 60% less membrane degradation respectively when compared with group A I. On day 7, Group B I at 5 mg/ml solution exhibited 48 % less protein release into the medium compared to group A I while which immersed in 20mg/ml, 50 mg/ml and 100 mg/ml exhibited 33%, 53% and 37% less membrane degradation respectively when compared with group A I. On day 14, Group B I at 5mg/ml, 20mg/ml and 100 mg/ml solution exhibited 9 % , 14%, 21 % more protein release into the medium compared to group A I while which immersed in 50 mg/ml exhibited 42 % less membrane degradation when compared with group A I.

Table 11, 12 & figure 3 shows mean values of free protein content of each of four different concentrations respectively in Group C treated with collagenase (C I) on Day 2, 4, 7 & 14. Results shows that at 5 mg/ml concentration, less new protein release in the beginning which increased till day 4 but decreased steadily up to day 14. At 20 mg/ml, new protein release increased steadily upto day 7 and then decreased at day 14. At 50mg/ml, there was not much difference in new protein release at day 4 & day 7 but new protein release decreased at day 14 whereas at 100 mg/ml, the new protein release increased steadily upto day 17 and then decreased at day 14 whereas mean values of free protein content of each of four different concentrations respectively in Group C treated without collagenase (C II) on Day 2, 4, 7 & 14 shows that at 5 mg/ml concentration, less new protein release in the beginning which increased till day 4 but decreased up to day 14. At 20 mg/ml, new protein release

increased steadily up to day 14. At 50mg/ml, there was steady increase in new protein up to day 14 while at 100 mg/ml, the new protein release increased at day 4 and then decreased to day 14.

In Group C I on Day 2, collagen membrane immersed at 5 mg/ml solution exhibited 73 % less protein release into the medium while which immersed in 20mg/ml, 50 mg/ml and 100 mg/ml exhibited 85%, 58% and 49% less membrane degradation respectively when compared with group A I. On day 4, Group C I at 5 mg/ml solution exhibited just 14 % less protein release into the medium while which immersed in 20mg/ml, 50 mg/ml and 100 mg/ml exhibited 60%, 60% and 45% less membrane degradation respectively when compared with group A I. On day 7, Group C I at 5 mg/ml solution exhibited 58 % less protein release into the medium compared to group A I while which immersed in 20mg/ml, 50 mg/ml and 100 mg/ml exhibited 66%, 70% and 60% less membrane degradation respectively when compared with group A I. On day 14, Group B I at 5mg/ml and 20mg/ml exhibited 80%, 89% less protein released into the medium compared to group A I while which immersed in 50 mg/ml and 100 mg/ml exhibited 75 % and 59 % less protein released when compared with group A I.

Table 13 shows the comparison between group B and group A in both with and without collagenase groups and also comparison between group C and group A in both with and without collagenase groups through One way ANOVA. The P values were 0.000 (< 0.001) for all the comparisons between group A I and BI and also between A II and B II except on day 4 and day 7 where P values are 0.006 (< 0.01) i.e. significant. The P values were 0.000 (< 0.001) for all the comparisons between group A I and C I except on day 4 where P value was not significant and also significant between A II and C II except on day 2 where P values are not significant.

Table 14 shows the results of multiple comparison test (Tukey HSD post hoc test) for free protein content for group A I and B I. The results shows that P values between different concentration of tetracycline compared with control group were statistically significant ($P < 0.001$) but P values for comparisons between different concentration of tetracycline group treated with collagenase were not significant.

Table 15 shows the results of multiple comparison test (Tukey HSD post hoc test) for free protein content for group A I and C I. The results shows that P values between different concentration of doxycyline compared with control group were statistically significant ($P < 0.001$) but P values for comparisons between different concentration of doxycyline group treated with collagenase were not significant.

Table 16 shows the results of independent T test for comparison between mean values of free protein content of group A I (control group with collagenase) and group A II (control group with collagenase) on different incubation periods. The results shows P values 0.000 (< 0.001) which is statistically significant.

Table 17 shows the results of independent T test for comparison between mean values of free protein content of group B I (tetracycline group with collagenase) and group B II (tetracycline group without collagenase) at varying concentrations on different incubation periods. The results shows P values (< 0.01) were statistically significant at 5 mg/ml on day 2,4 and 7 while on day 14 P values were not significant. At 20 mg/ml only on day 14, P values are statistically significant. At 50 mg/ml, P values (< 0.001) were statistically significant on all the days. At 100 mg/ml, P values (< 0.01) were statistically significant.

Table 18 shows the results of independent T test for comparison between mean values of free protein content of group C I (doxycyline group with collagenase) and

group B II (doxycyline group without collagenase) at varying concentrations on different incubation periods. The results shows P values (< 0.05) were statistically significant at 5 mg/ml on day 2,4 and 7 while on day 14 P values were not significant. At 20 mg/ml, P values (< 0.05) were statistically significant. At 50 mg/ml, P values (< 0.01) were statistically significant on day 2 and 14 but the values are not significant on day 4,7. At 100 mg/ml, P values (< 0.05) were statistically significant on day 2 while P values (< 0.01) were statistically significant on day 4 and 7 but not significant on day 14.

Photomicrograph 1,2,3,4 shows microscopic view of remaining collagen membrane in medium containing Phosphate buffer solution treated with collagenase (group A I). Loss of collagen structure was observed from day 2 which continue up to day 14 when it was observed as almost complete loss of collagen membrane leaving behind few collagen fibers.

Photomicrograph 5 shows microscopic view of remaining collagen membrane in medium containing group B I at 5 mg/ml concentration over different incubation periods. On day 2 the collagen membrane was appeared to be structurally disorganized which continue to loss the structure over the time up to day 14 when small collagen fibers were left in the medium.

Photomicrograph 6 shows microscopic view of remaining collagen membrane in medium containing group B I at 20 mg/ml concentration over different incubation periods. Destruction of collagen fibers were observed from day 2 which continue to increased up to day 14 where disorganized collagen fibers were seen.

Photomicrograph 7 shows microscopic view of remaining collagen membrane in medium containing group B I at 50 mg/ml concentration over different incubation

periods. No change in collagen structure were observed on day 2 and day 4 but the degeneration collagen fibers were first observed on day 7 with not much increased loss of collagen on day 14.

Photomicrograph 8 shows microscopic view of remaining collagen membrane in medium containing group B I at 100 mg/ml concentration over different incubation periods. Destruction of collagen fibers were observed from day 7 which was found to be maximum on day 14

Photomicrograph 9 shows microscopic view of remaining collagen membrane in medium containing group C I at 5 mg/ml concentration over different incubation periods. No change in the structure of collagen membrane was found upto day 4. It was first appeared to be structurally disorganized on day 7which continue to loss the structure over the time up to day 14

Photomicrograph 10 shows microscopic view of remaining collagen membrane in medium containing group C I at 20 mg/ml concentration over different incubation periods. No change in the structure of collagen membrane was observed up to day 14.

Photomicrograph 11 shows microscopic view of remaining collagen membrane in medium containing group C I at 50 mg/ml concentration over different incubation periods. No change in collagen structure were observed on day 2 but the disorganized collagen fibers were first observed on day 4 which was seen slightly more on day 14.

Photomicrograph 12 shows microscopic view of remaining collagen membrane in medium containing group C I at 100 mg/ml concentration over different incubation periods. Destruction of collagen fibers were observed from day 7 which was found to be more on day 14.

Table 1:

Free Protein content in Group A-I

MASTER CHART

S.No	Day 2	Day 4	Day 7	Day 14
1	0.211	0.246	0.399	0.284
2	0.285	0.313	0.364	0.291
3	0.301	0.351	0.482	0.234
4	0.199	0.212	0.298	0.213
5	0.264	0.29	0.406	0.25
6	0.324	0.328	0.461	0.228

Table 2:

Free Protein content in Group A -II

MASTER CHART

S.No.	Day 2	Day 4	Day 7	Day 14
1	0.016	0.056	0.122	0.158
2	0.011	0.042	0.137	0.162
3	0.031	0.068	0.169	0.235
4	0.024	0.071	0.151	0.193
5	0.029	0.089	0.179	0.201
6	0.032	0.100	0.148	0.209

Table 3:

Free Protein content in Group B- I

MASTER CHART

CONCENTRATION	Day 2	Day 4	Day 7	Day 14
5mg/ml	0.145	0.153	0.209	0.273
	0.134	0.14	0.158	0.219
	0.151	0.166	0.192	0.208
	0.158	0.161	0.248	0.312
	0.131	0.144	0.212	0.341
	0.154	0.158	0.236	0.288
20mg/ml	0.138	0.118	0.322	0.348
	0.152	0.126	0.298	0.375
	0.145	0.122	0.269	0.304
	0.132	0.113	0.23	0.286
	0.115	0.094	0.175	0.256
	0.113	0.109	0.089	0.151
50mg/ml	0.128	0.166	0.166	0.258
	0.122	0.112	0.136	0.104
	0.184	0.132	0.19	0.145
	0.212	0.128	0.202	0.132
	0.232	0.136	0.212	0.124
	0.226	0.122	0.231	0.107
100mg/ml	0.123	0.100	0.321	0.328
	0.117	0.095	0.155	0.205
	0.105	0.050	0.213	0.299
	0.162	0.115	0.255	0.304
	0.242	0.105	0.299	0.256
	0.223	0.225	0.287	0.432

Table 4:

Free Protein content in Group B- II

MASTER CHART

CONCENTRATION	Day 2	Day 4	Day 7	Day 14
5mg/ml	0.01	0.081	0.039	0.058
	0.006	0.06	0.042	0.112
	0.014	0.075	0.053	0.094
	0.024	0.079	0.075	0.108
	0.012	0.075	0.038	0.119
	0.018	0.08	0.071	0.073
20mg/ml	0.024	0.079	0.156	0.165
	0.032	0.062	0.181	0.128
	0.021	0.052	0.142	0.142
	0.016	0.078	0.199	0.114
	0.054	0.042	0.203	0.114
	0.045	0.058	0.205	0.105
50mg/ml	0.025	0.052	0.054	0.037
	0.032	0.066	0.043	0.051
	0.081	0.075	0.032	0.053
	0.051	0.061	0.051	0.042
	0.047	0.072	0.035	0.065
	0.046	0.070	0.043	0.058
100mg/ml	0.036	0.053	0.141	0.201
	0.047	0.051	0.115	0.186
	0.029	0.065	0.128	0.178
	0.021	0.037	0.156	0.217
	0.037	0.058	0.155	0.191
	0.012	0.042	0.151	0.233

Table 5:

Free Protein content in Group C- I

MASTER CHART

CONCENTRATION	Day 2	Day 4	Day 7	Day 14
5mg/ml	0.1	0.074	0.094	0.16
	0.102	0.122	0.159	0.023
	0.072	0.103	0.168	0.05
	0.042	0.086	0.184	0.032
	0.053	0.136	0.212	0.019
	0.064	0.98	192	0.016
20mg/ml	0.078	0.121	0.161	0.022
	0.038	0.112	0.135	0.029
	0.064	0.079	0.031	0.088
	0.022	0.152	0.212	0.013
	0.018	0.116	0.193	0.016
	0.015	0.092	0.078	0.006
50mg/ml	0.1	0.077	0.129	0.049
	0.101	0.089	0.038	0.157
	0.042	0.158	0.128	0.025
	0.062	0.104	0.118	0.064
	0.037	0.134	0.152	0.053
	0.031	0.062	0.143	0.036
100mg/ml	0.155	0.213	0.199	0.076
	0.076	0.151	0.162	0.103
	0.025	0.124	0.126	0.053
	0.145	0.171	0.174	0.243
	0.041	0.151	0.165	0.107
	0.014	0.151	0.146	0.036

Table 6:

Free Protein content in Group C-II

MASTER TABLE

CONCENTRATION	Day 2	Day 4	Day 7	Day 14
5mg/ml	0.026	0.201	0.1	0.062
	0.013	0.116	0.151	0.093
	0.02	0.144	0.118	0.1
	0.031	0.132	0.122	0.128
	0.011	0.141	0.123	0.141
	0.019	0.13	0.094	0.076
20mg/ml	0.013	0.011	0.050	0.059
	0.008	0.014	0.030	0.070
	0.019	0.023	0.041	0.062
	0.035	0.030	0.039	0.051
	0.015	0.025	0.052	0.074
	0.024	0.035	0.034	0.056
50mg/ml	0.009	0.038	0.067	0.128
	0.014	0.069	0.093	0.152
	0.011	0.049	0.076	0.133
	0.016	0.101	0.123	0.197
	0.015	0.073	0.107	0.121
	0.019	0.084	0.092	0.181
100mg/ml	0.009	0.094	0.076	0.054
	0.015	0.114	0.1	0.081
	0.011	0.109	0.09	0.061
	0.021	0.138	0.112	0.099
	0.013	0.1	0.084	0.078
	0.021	0.129	0.138	0.113

Table 7:

MEAN VALUES OF FREE PROTEIN CONTENT in GROUP A- I

Incubation period	Number of samples	Mean	Standard deviation
Day 2	6	0.264	0.049
Day 4	6	0.290	0.052
Day 7	6	0.401	0.066
Day 14	6	0.250	0.031

Table 8:

MEAN VALUES OF FREE PROTEIN CONTENT IN GROUP A- II

Incubation period	Number of samples	Mean	Standard deviation
Day 2	6	0.023	0.008
Day 4	6	0.071	0.021
Day 7	6	0.151	0.020
Day 14	6	0.193	0.029

Table 9:

MEAN VALUES OF FREE PROTEIN CONTENT in GROUP B-I

Concentration	Incubation period	Number of samples	Mean	Standard deviation
5mg/ml	**Day 2**	6	0.145	0.010
	Day 4	6	0.153	0.010
	Day 7	6	0.209	0.032
	Day 14	6	0.273	0.051
20 mg/ml	**Day 2**	6	0.132	0.015
	Day 4	6	0.113	0.011
	Day 7	6	0.230	0.086
	Day 14	6	0.286	0.079
50 mg/ml	**Day 2**	6	0.184	0.048
	Day 4	6	0.132	0.018
	Day 7	6	0.189	0.034
	Day 14	6	0.145	0.057
100mg/ml	**Day 2**	6	0.162	0.058
	Day 4	6	0.115	0.058
	Day 7	6	0.255	0.061
	Day 14	6	0.304	0.074

Table 10:

MEAN VALUES OF FREE PROTEIN CONTENT in GROUP B-II

Concentration	Incubation period	Number of samples	Mean	Standard deviation
5mg/ml	**Day 2**	6	0.014	0.006
	Day 4	6	0.075	0.007
	Day 7	6	0.053	0.016
	Day 14	6	0.094	0.024
20mg/ml	**Day 2**	6	0.032	0.014
	Day 4	6	0.061	0.014
	Day 7	6	0.181	0.026
	Day 14	6	0.128	0.022
50g/ml	**Day 2**	6	0.047	0.019
	Day 4	6	0.066	0.026
	Day 7	6	0.043	0.036
	Day 14	6	0.051	0.036
100mg/ml	**Day 2**	6	0.030	0.009
	Day 4	6	0.051	0.022
	Day 7	6	0.141	0.039
	Day 14	6	0.201	0.051

Table 11:

MEAN VALUES OF FREE PROTEIN CONTENT in GROUP C –I

Concentration	Incubation period	Number of samples	Mean	Standard deviation
5mg/ml	**Day 2**	6	0.072	0.024
	Day 4	6	0.250	0.358
	Day 7	6	0.168	0.040
	Day 14	6	0.050	0.055
20mg/ml	**Day 2**	6	0.039	0.026
	Day 4	6	0.112	0.025
	Day 7	6	0.135	0.069
	Day 14	6	0.029	0.029
50mg/ml	**Day 2**	6	0.062	0.031
	Day 4	6	0.114	0.036
	Day 7	6	0.118	0.040
	Day 14	6	0.064	0.047
100mg/ml	**Day 2**	6	0.076	0.061
	Day 4	6	0.160	0.029
	Day 7	6	0.162	0.024
	Day 14	6	0.103	0.073

Table 12:

MEAN VALUES OF FREE PROTEIN CONTENT in GROUP C-II

concentration	Incubation period	Number of samples	Mean	Standard deviation
5mg/ml	**Day 2**	6	0.020	0.007
	Day 4	6	0.144	0.029
	Day 7	6	0.118	0.020
	Day 14	6	0.100	0.030
20mg/ml	**Day 2**	6	0.019	0.009
	Day 4	6	0.023	0.009
	Day 7	6	0.041	0.008
	Day 14	6	0.062	0.008
50mg/ml	**Day 2**	6	0.014	0.003
	Day 4	6	0.069	0.022
	Day 7	6	0.093	0.020
	Day 14	6	0.152	0.030
100mg/ml	**Day 2**	6	0.015	0.050
	Day 4	6	0.114	0.016
	Day 7	6	0.100	0.022
	Day 14	6	0.081	0.022

Table 13:

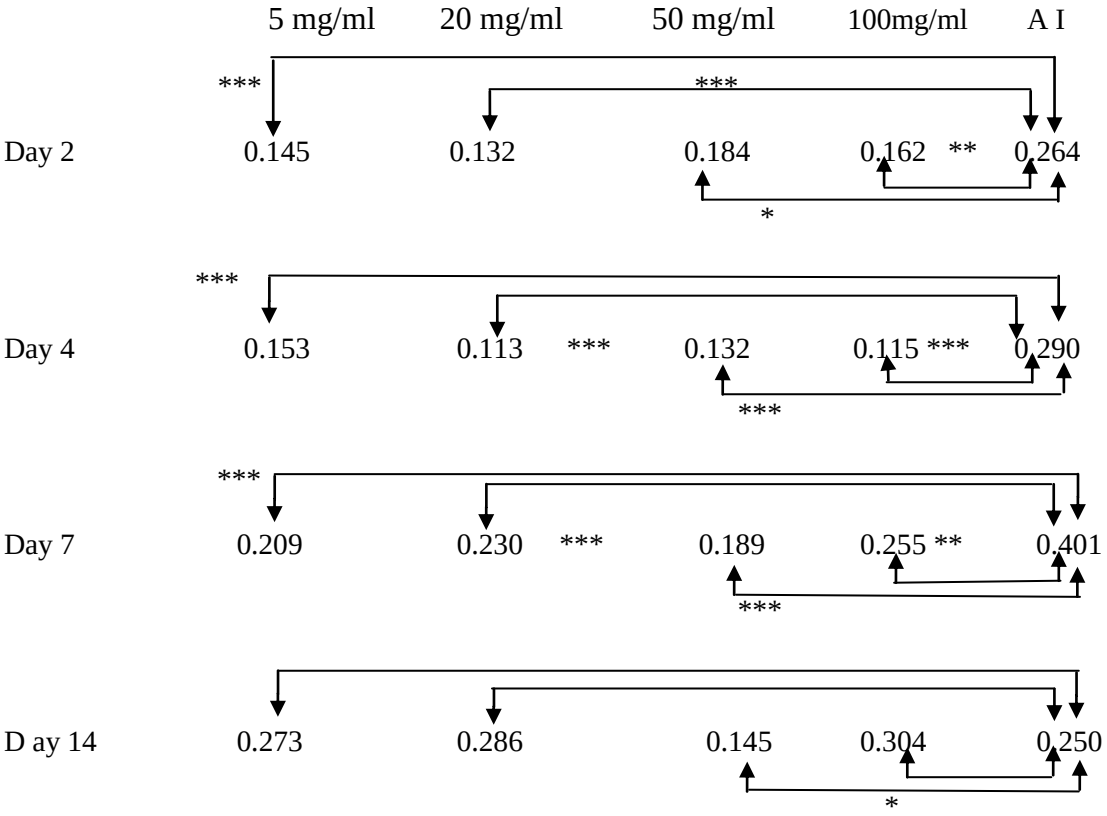
ONE WAY ANOVA FOR BETWEEN GROUP COMPARISONS

	GROUP	BETWEEN GROUPS	SIGNIFICANCE
Protein content at Day 2	A I	B I	**0.000**
	B I		
Protein content at Day 4	A I	B I	**0.000**
	B I		
Protein content at Day 7	A I	B I	**0.000**
	B I		
Protein content at Day 14	A I	B I	**0.001**
	B I		
Protein content at Day 2	A II	B II	**0.006**
	B II		
Protein content at Day 4	A II	B II	**0.007**
	B II		
Protein content at Day 7	A II	B II	**0.000**
	B II		
Protein content at Day 14	A II	B II	**0.000**
	B II		
Protein content at Day 2	A I	C I	**0.000**
	C I		
Protein content at Day 4	A I	C I	**0.192**
	C I		
Protein content at Day 7	A I	C I	**0.000**
	C I		
Protein content at Day 14	A I	C I	**0.000**
	C I		
Protein content at Day 2	A II	C II	**0.156**
	C II		
Protein content at Day 4	A II	C II	**0.000**
	C II		
Protein content at Day 7	A II	C II	**0.000**
	C II		
Protein content at Day 14	A II	C II	**0.000**
	C II		

Table 14:

MULTIPLE COMPARISON POST HOC TESTS

PROTEIN CONTENT IN MEDIUM FOR GROUP B I & A I



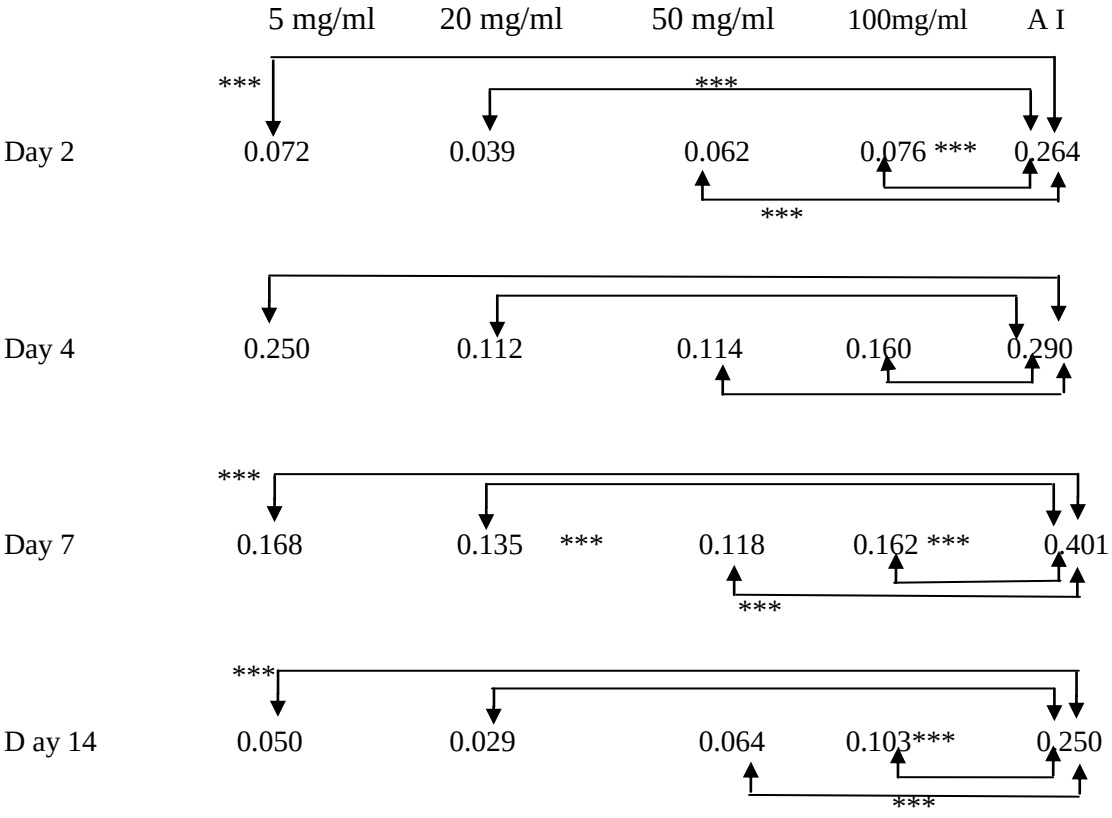
The results are presented as mean ± standard deviation

P < 0.05 *, P < 0.01 **, P < 0.001 ***

Table 15:

MULTIPLE COMPARISON POST HOC TESTS

PROTEIN CONTENT IN MEDIUM FOR GROUP C I & A I



The results are presented as mean ± standard deviation

P < 0.05 *, P < 0.01 **, P < 0.001 ***

Table 16:

INDEPENDENT T TEST - GROUP A I Vs A II

	Incubation period	Group	Group	No. of samples	Significance
BSA	**Day 2**	A	I	6	**0.000**
			II	6	
	Day 4	A	I	6	**0.000**
			II	6	
	Day 7	A	I	6	**0.000**
			II	6	
	Day 14	A	I	6	**0.000**
			II	6	

Table 17:

INDEPENDENT T TEST – Group B I Vs B II

Concentration	Incubation period	Group	Group	No. of samples	Significance
5 mg/ml	Day 2	B	I	6	0.001
			II	6	
	Day 4	B	I	6	0.048
			II	6	
	Day 7	B	I	6	0.022
			II	6	
	Day 14	B	I	6	0.080
			II	6	
20 mg/ml	Day 2	B	I	6	0.108
			II	6	
	Day 4	B	I	6	0.223
			II	6	
	Day 7	B	I	6	0.263
			II	6	
	Day 14	B	I	6	0.000
			II	6	
50 mg/ml	Day 2	B	I	6	0.000
			II	6	
	Day 4	B	I	6	0.000
			II	6	
	Day 7	B	I	6	0.000
			II	6	
	Day 14	B	I	6	0.003
			II	6	
100mg/ml	Day 2	B	I	6	0.000
			II	6	
	Day 4	B	I	6	0.025
			II	6	
	Day 7	B	I	6	0.001
			II	6	
	Day 14	B	I	6	0.010
			II	6	

Table 18:

INDEPENDENT T TEST – Group C I vs C II

Concentration	Incubation period	Group	Group	No. of samples	Significance
5 mg/ml	**Day 2**	C	I	6	**0.001**
			II	6	
	Day 4	C	I	6	**0.048**
			II	6	
	Day 7	C	I	6	**0.022**
			II	6	
	Day 14	C	I	6	**0.080**
			II	6	
20 g/ml	**Day 2**	C	I	6	**0.01**
			II	6	
	Day 4	C	I	6	**0.02**
			II	6	
	Day 7	C	I	6	**0.02**
			II	6	
	Day 14	C	I	6	**0.01**
			II	6	
50 mg/ml	**Day 2**	C	I	6	**0.004**
			II	6	
	Day 4	C	I	6	**0.073**
			II	6	
	Day 7	C	I	6	**0.210**
			II	6	
	Day 14	C	I	6	**0.003**
			II	6	
100mg/ml	**Day 2**	C	I	6	**0.035**
			II	6	
	Day 4	C	I	6	**0.008**
			II	6	
	Day 7	C	I	6	**0.001**
			II	6	
	Day 14	C	I	6	**0.501**
			II	6	

Table 19:

Independent T test: Comparison between group B 50 mg/ml and group C 20 mg/ml with collagenase

	Incubation period	Group	Group	No. of samples	Significance
Free Protein content in the medium	**Day 2**	B 50 mg/ml	I	6	**0.000**
		C 20 mg/ml		6	
	Day 4	B 50 mg/ml	I	6	**0.115**
		C 20 mg/ml		6	
	Day 7	B 50 mg/ml	I	6	**0.136**
		C 20 mg/ml		6	
	Day 14	B 50 mg/ml	I	6	**0.001**
		C 20 mg/ml		6	

Figure 1: Comparison of mean values of free protein content in medium (optical density @ 595nm) between group A I and A II

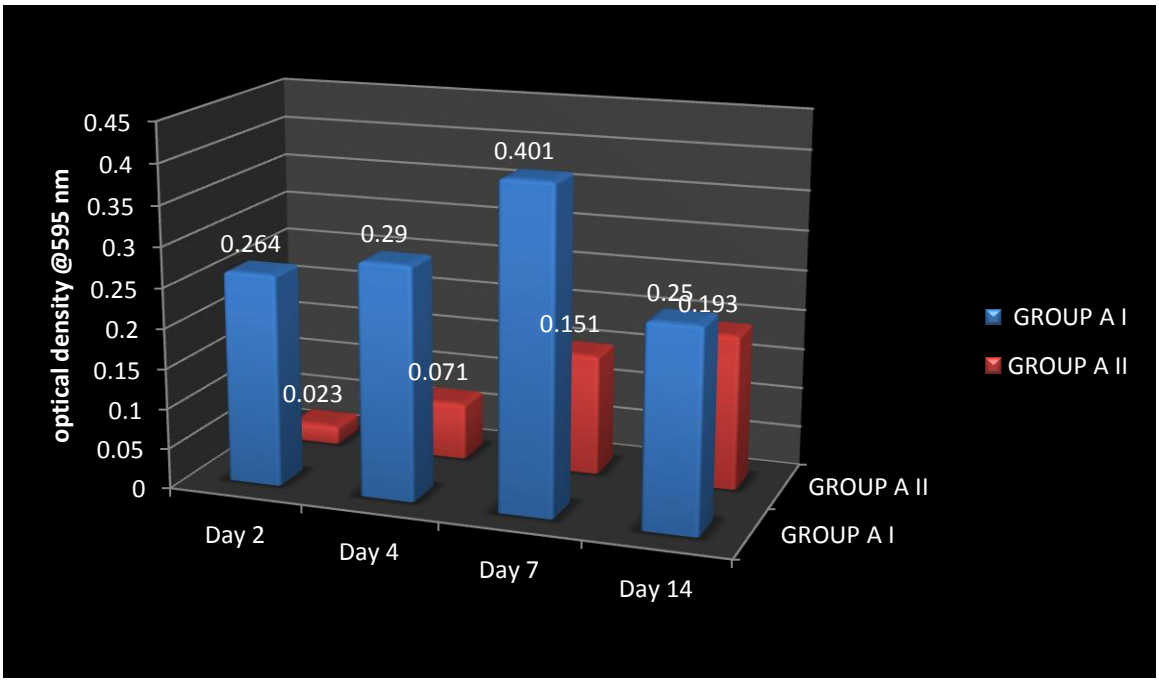


Figure 2: Comparison of mean values of free protein content in medium (optical density @ 595nm) between group B I and B II

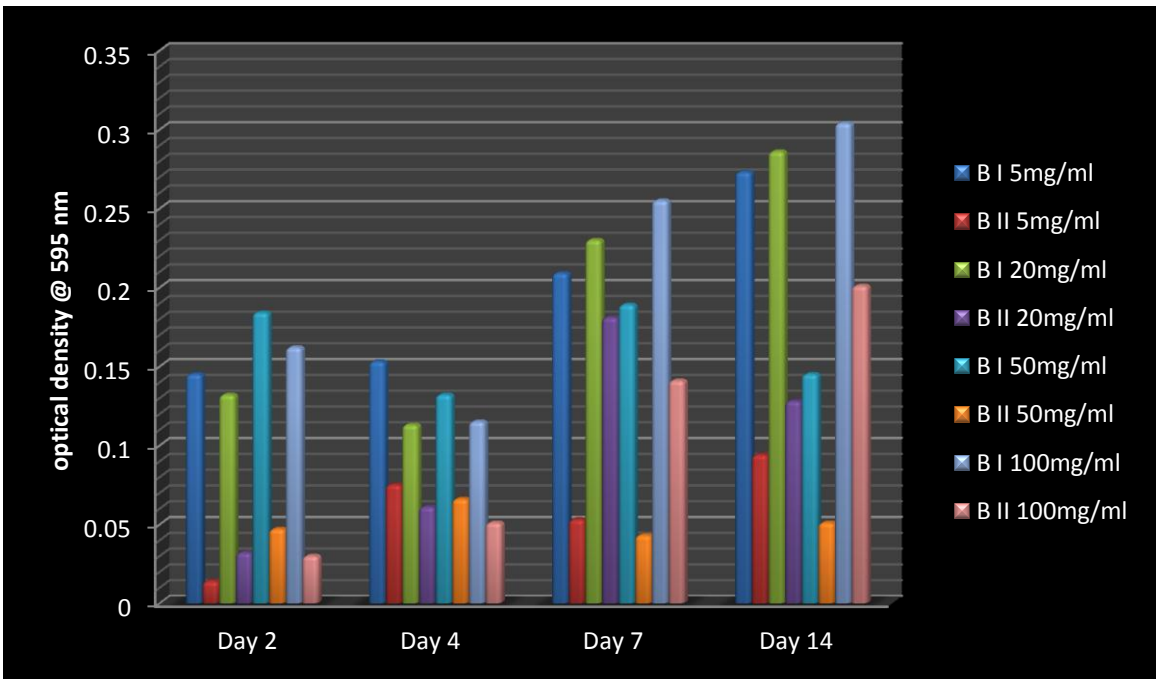


Figure 3: Comparison of mean values of free protein content in medium (optical density @ 595nm) between group C I and C II

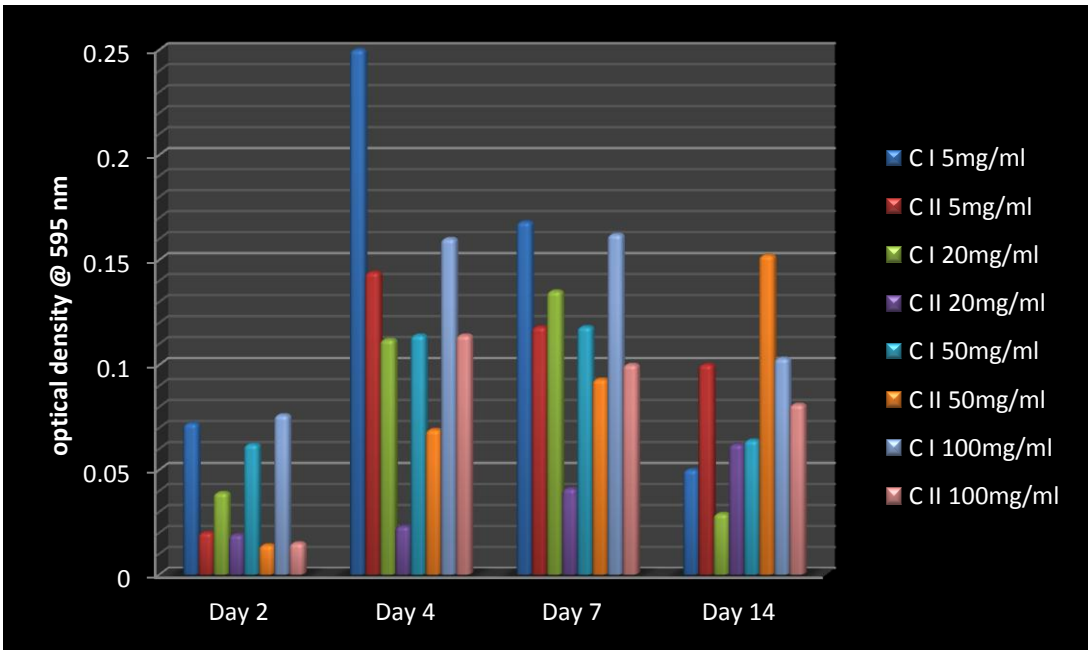


Figure 4: Comparison of mean values of free protein content in medium (optical density @ 595nm) between group A I and B I

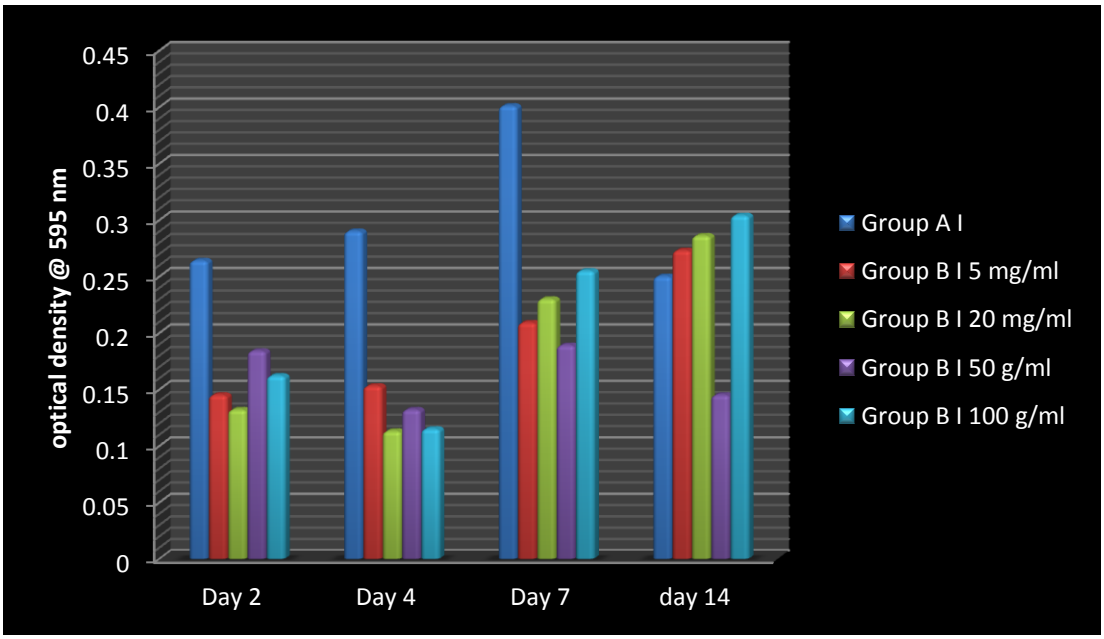


Figure 5: Comparison of mean values of free protein content in medium (optical density @ 595nm) between group A I and C I

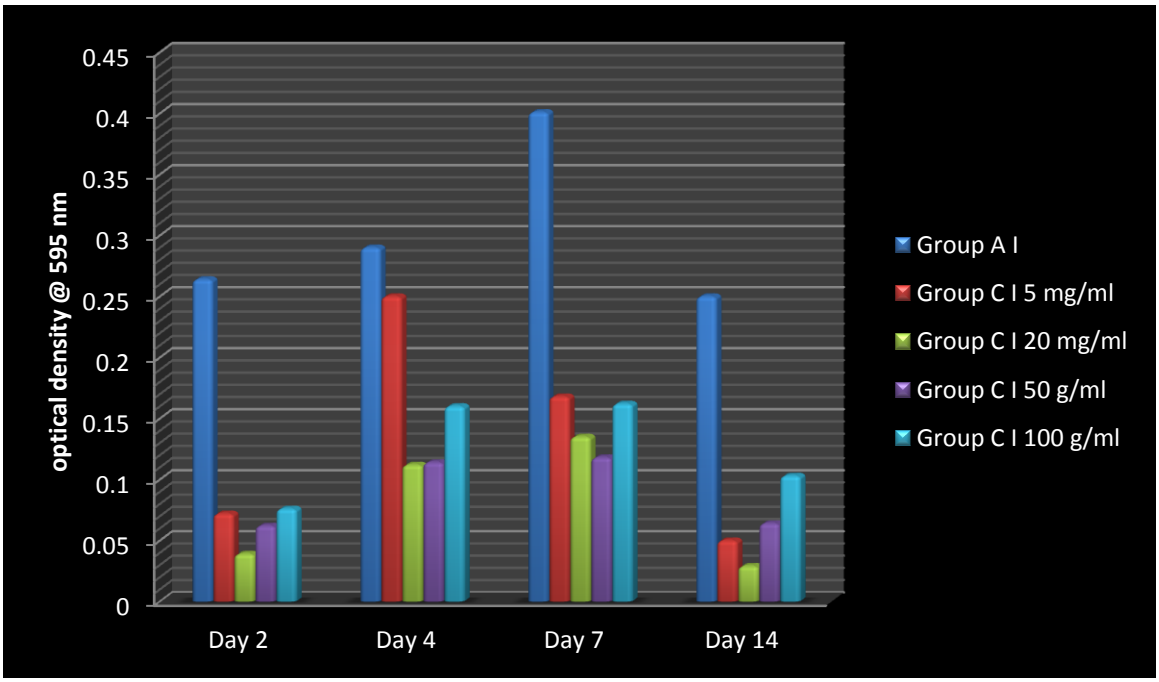
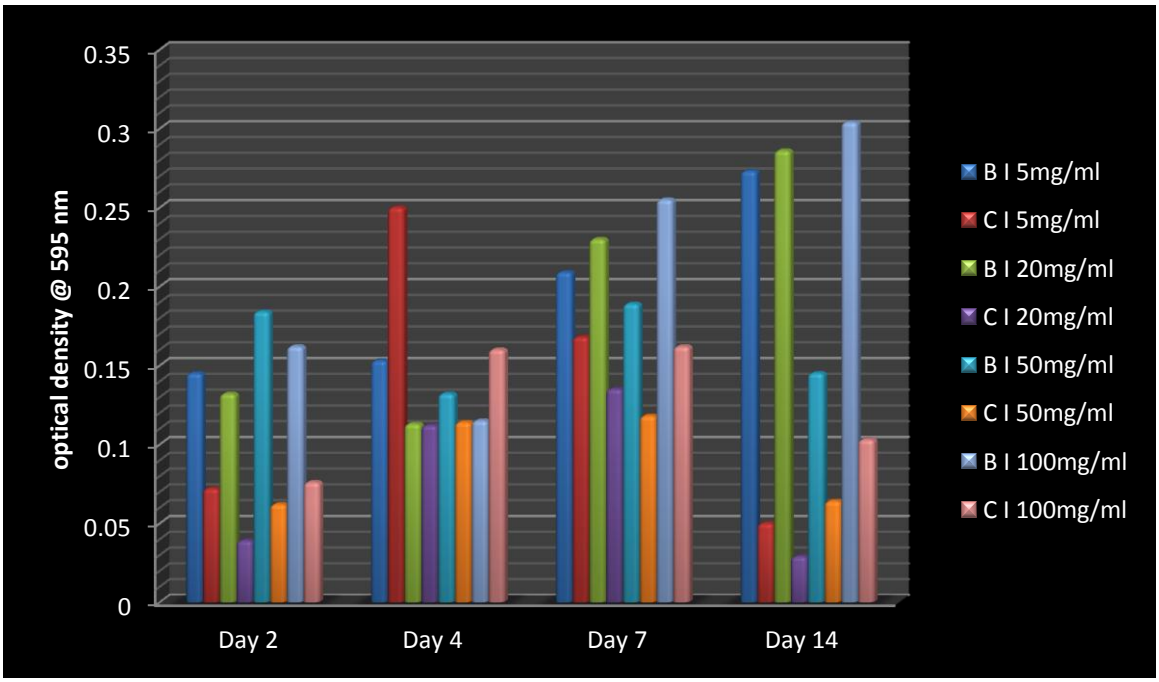
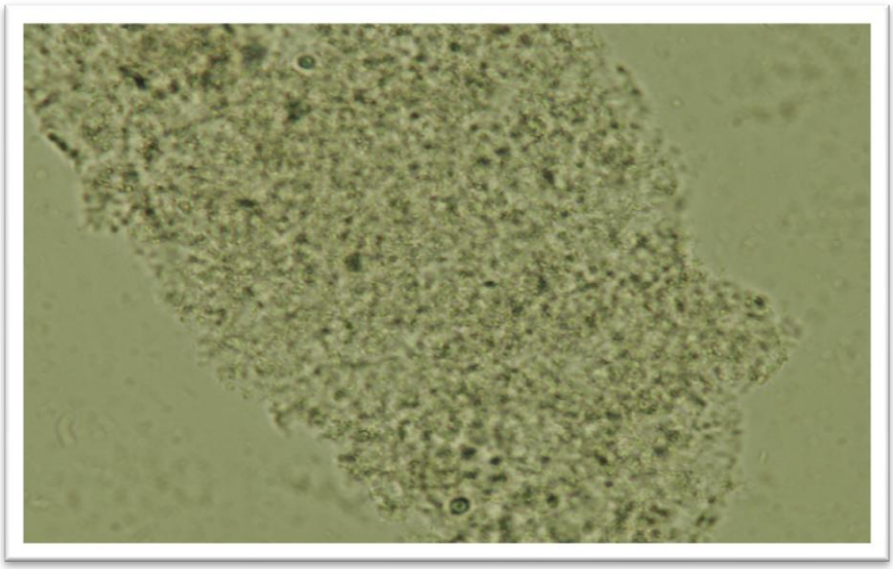


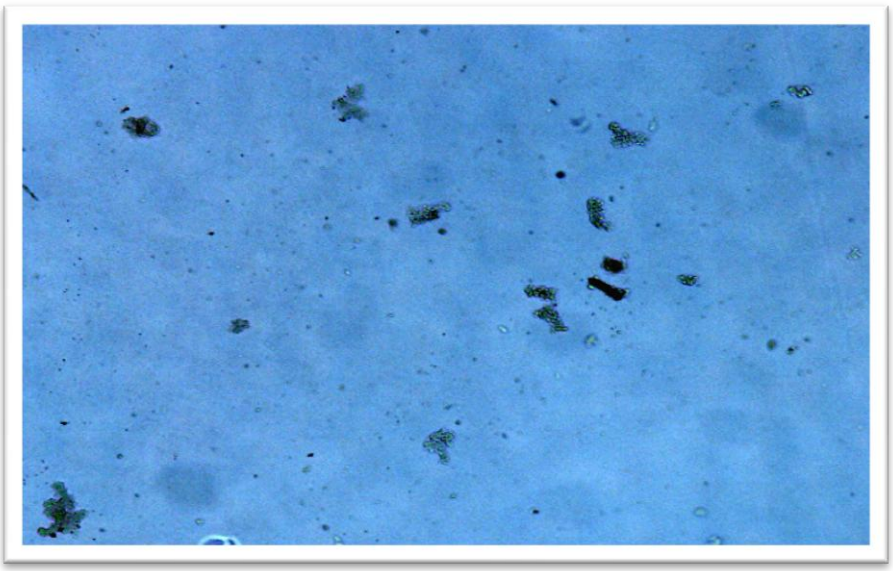
Figure 6: Comparison of mean values of free protein content in medium (optical density @ 595nm) between group B I and C I



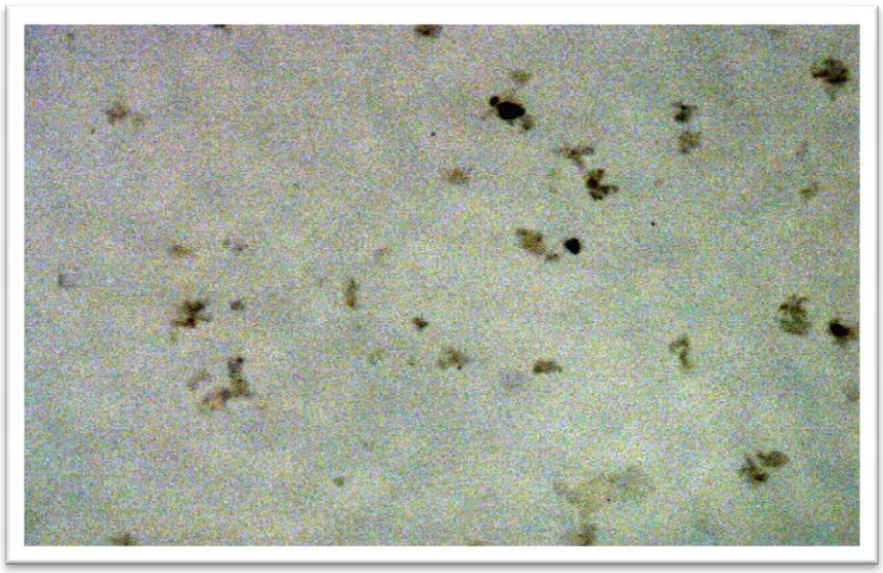
PHOTOMICROGRAPH 1: GROUP A- I 100 X MAGNIFICATION (DAY 2)



PHOTOMICROGRAPH 2: GROUP A – I 40 X MAGNIFICATION (DAY 4)

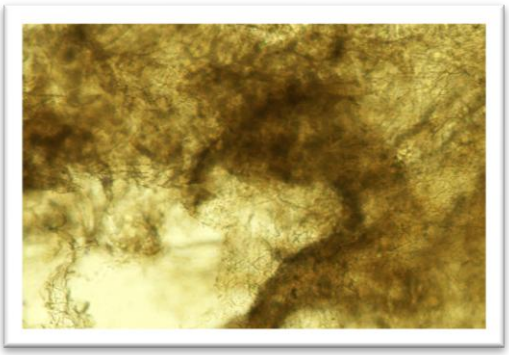


PHOTOMICROGRAPH 3: GROUP A- I 40 X MAGNIFICATION (DAY7)

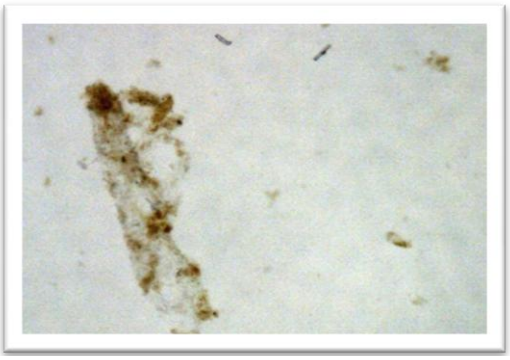


PHOTOMICROGRAPH 4: GROUP A- I 100 X MAGNIFICATION (DAY 14)

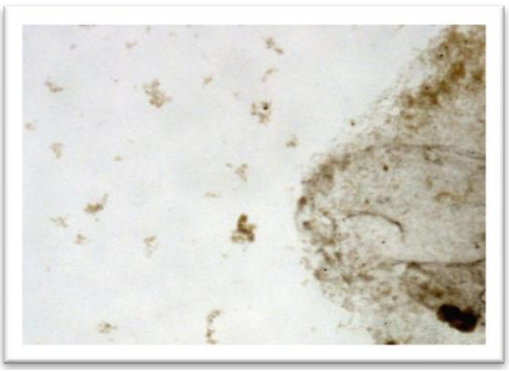
PHOTOMICROGRAPH 5: GROUPB- I (5mg/ml)



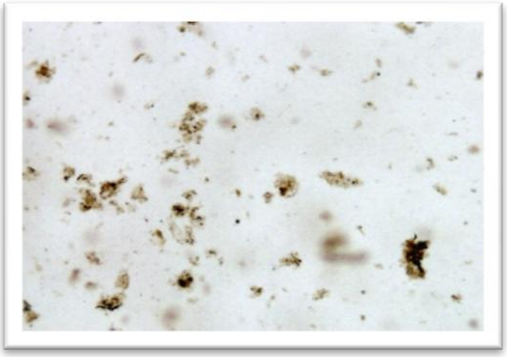
(i) 100 X Magnification (Day 2)



(ii) 40 X Magnification (Day 4)

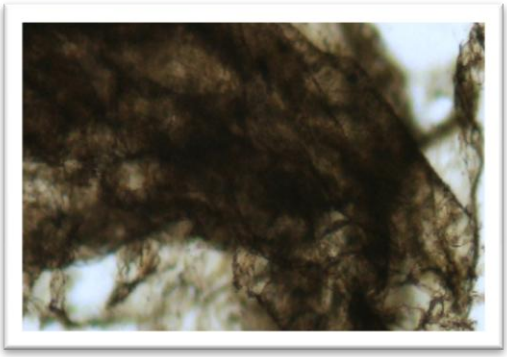


(iii) 100 X Magnification (Day 7)

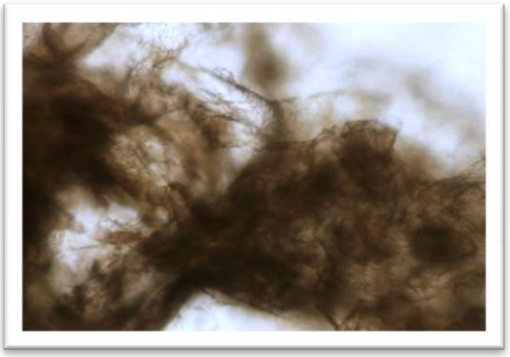


(iv) 40 X Magnification (Day 14)

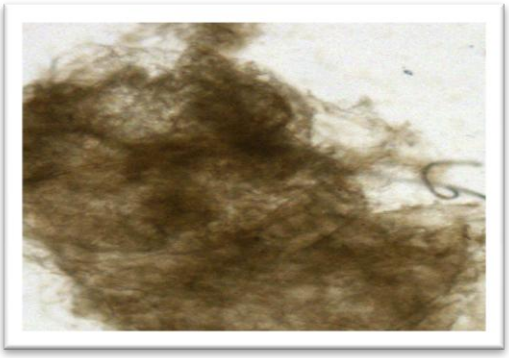
PHOTOMICROGRAPH 6 : GROUP B – I (20mg/ml)



(i) 100 X Magnification (Day 2)



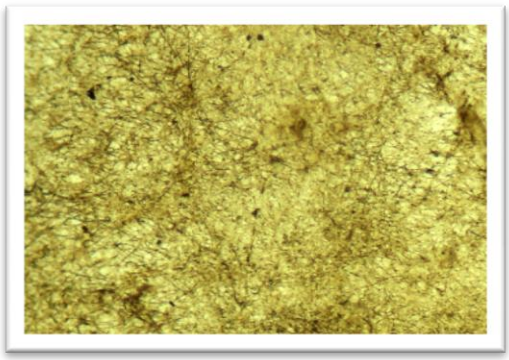
(ii) 100 X Magnification (Day 4)



(iii) 100 X Magnification (Day 7)

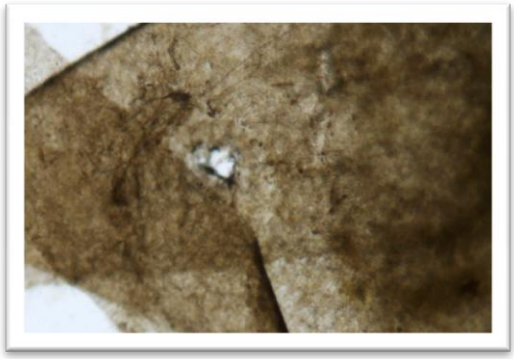
(iv) 100 X Magnification (Day 14)

PHOTOMICROGRAPH 7: GROUP B – I (50mg/ml)

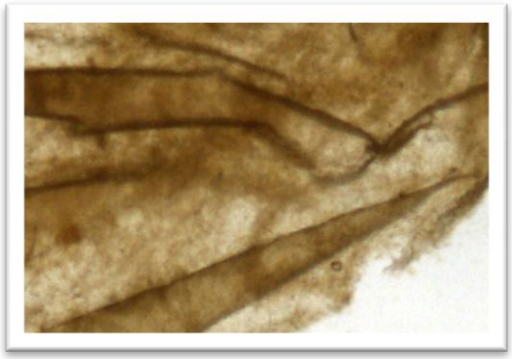


(i) 100 X Magnification (Day 2)

(ii) 100 X Magnification (Day 4)

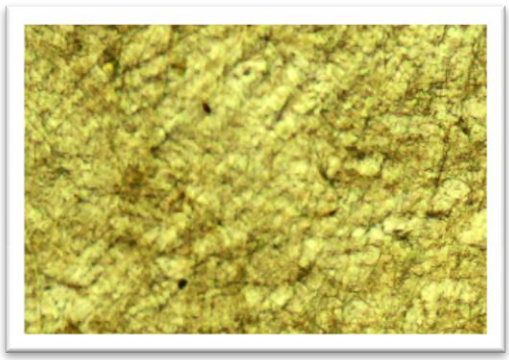


(iii) 100 X Magnification (Day 7)



(iv) 100 X Magnification (Day 14)

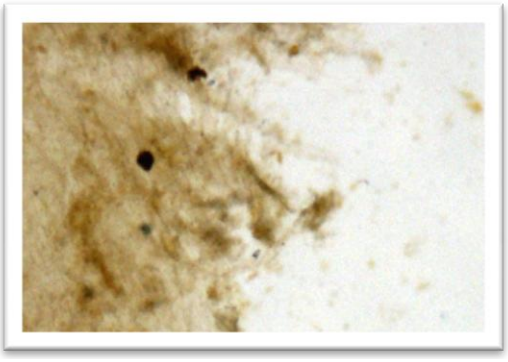
PHOTOMICROGRAPH 8: GROUP B –I (100mg/ml)



(i) 100 X Magnification (Day 2)

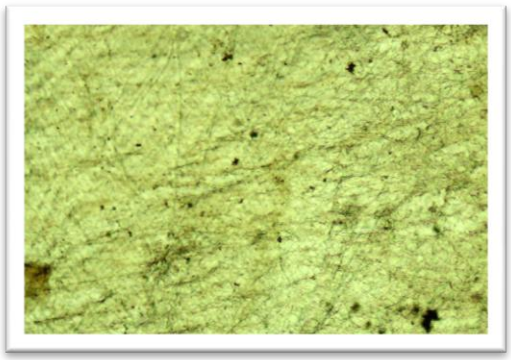
(ii) 100 X Magnification (Day 4)

(iii) 100 X Magnification (Day 7)



(iv) 100 X Magnification (Day 14)

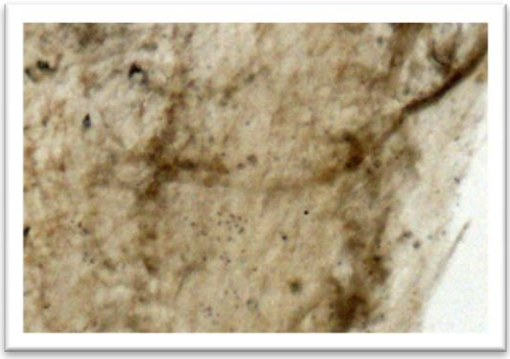
PHOTOMICROGRAPH 9: GROUP C- I (5mg/ml)



(i) 100 X Magnification (Day 2)

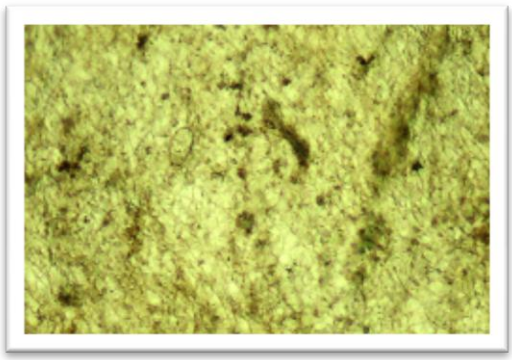
(ii) 100 X Magnification (Day 4)

(iii) 100 X Magnification (Day 7)

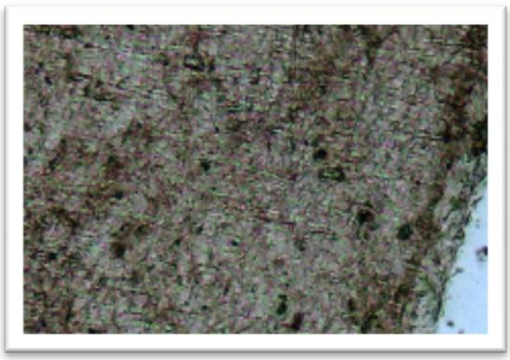


(iv) 100 X Magnification (Day 14)

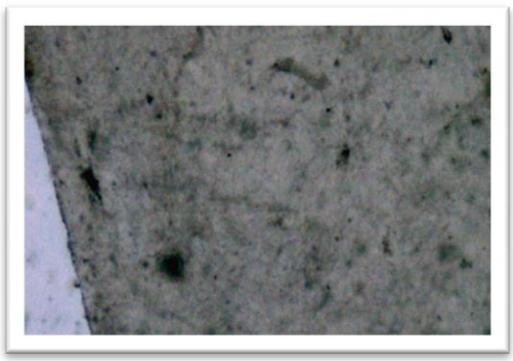
PHOTOMICROGRAPH 10: GROUP C – I (20mg/ml)



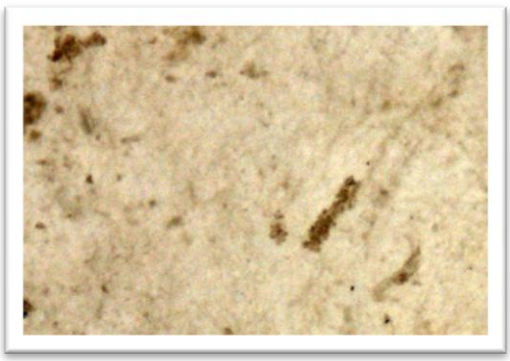
(i) 100 X Magnification (Day 2)



(ii) 100 X Magnification (Day 4)

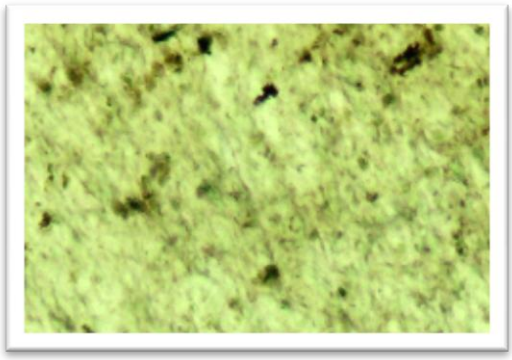


(iii) 100 X Magnification (Day 7)

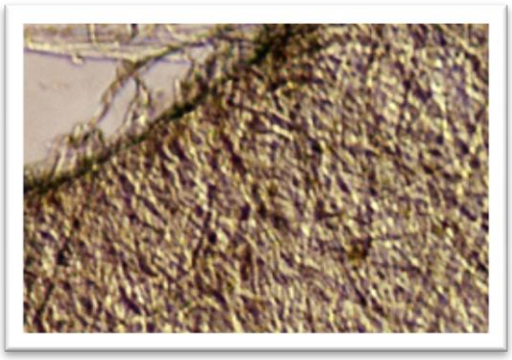


(iv) 100 X Magnification (Day 14)

PHOTOMICROGRAPH 11: GROUP C- I (50mg/ml)



(i) 100 X Magnification (Day 2)



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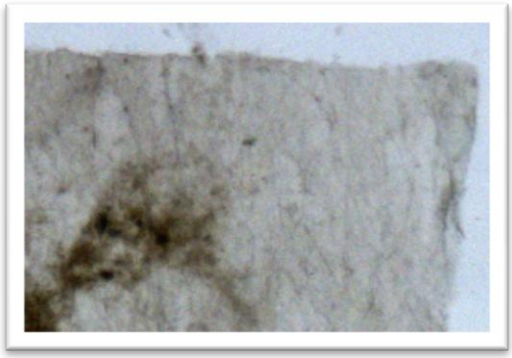
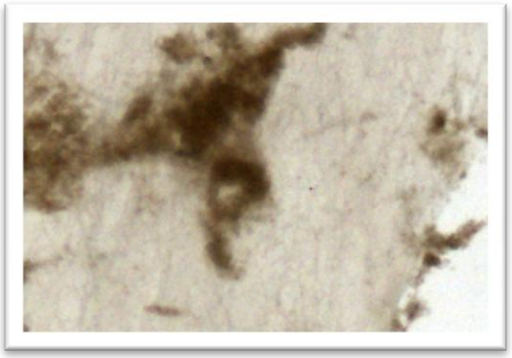
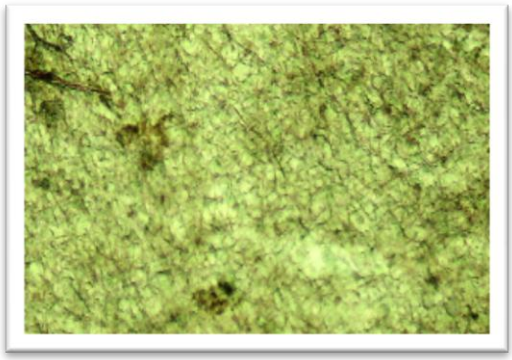


(iii) 100 X Magnification (Day 7

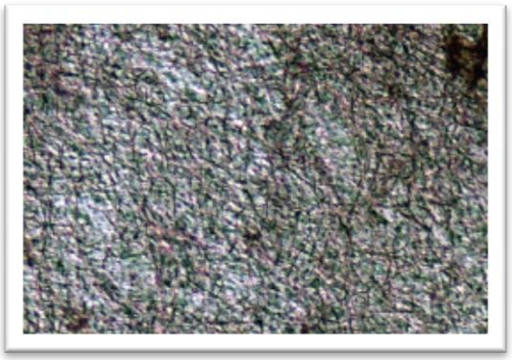


(iv) 100 X Magnification (Day 14)

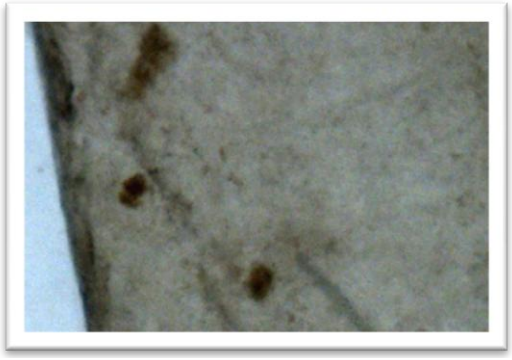
PHOTOMICROGRAPH 12: GROUP C- I (100mg/ml)



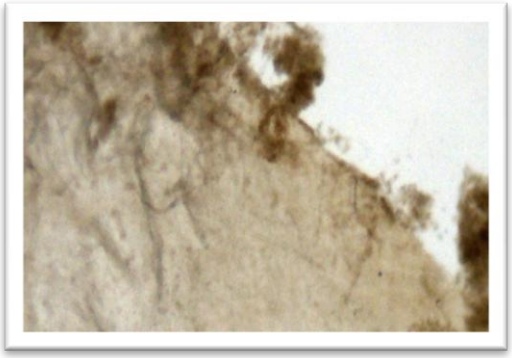
(i) 100 X Magnification (Day 2)



(ii) 100 X Magnification (Day 4)



(iii) 100 X Magnification (Day 7)



(iv) 100 X Magnification (Day 14)

DISCUSSION

Collagen comprises the most substantial group of structural proteins in connective tissue and it represents about one third of total body proteins. The rationale behind using collagen as a barrier is, as it is an extracellular macromolecule for periodontal connective tissue and is physiologically metabolized; it is chemotactic for fibroblasts, hemostatic and a weak immunogen and scaffolding for migrating cells.

Collagenase is thought to play a major role in the enzymatic degradation of collagenous materials. If the collagen membranes are prematurely resorbed, their barrier effect may be significantly reduced[26,67]. As the structural integrity of the collagen membrane for a sufficient period of time is essential for the success of guided tissue regeneration procedure, degradation time of bioresorbable collagen membranes should be prolonged to get better results.

Various materials have been used to control the rate of bioresorbable collagen membranes degradation. Tetracycline and its semisynthetic analogue doxycycline are the compounds which possess antibacterial and anticollagenolytic properties[51]. So tetracycline prolongs collagen membrane degradation by a local effect on tissue and anticollagenolytic effect

Ofer Moses et al [53] recently proved that collagen membranes immersed in TTC solution prior to their implantation and systemic administration of TTC significantly decreased the membranes degradation. Also Ofer Moses et al[54] proved that collagen membranes immersed in 50 mg/ml tetracycline hydrochloride solution exhibited the longest degradation time, both in clostridial collagenase and human bone lineage cell assays in vitro.

In the present study, we analyzed the effects of bioresorbable collagen membranes on their rate of degradation by clostridial collagenase when immersed in varying concentration of tetracycline and doxycycline in vitro by spectrophotometric analysis (quantitatively) and optical microscopic analysis (qualitatively).

Our findings showed that both tetracycline and doxycycline delays the degradation of collagen membranes differently at varying concentrations.

Quantitative analysis revealed that protein release in the medium analyzed by spectrophotometer at optical density @ 595nm was less at higher concentration of tetracycline while it was less at lower concentration of doxycycline.

This may indicate that more collagen degradation was observed in solution containing lower concentration of tetracycline whereas more collagen degradation was observed in solution containing higher concentrations of doxycycline. Also it was found that tetracycline at 50 mg/ml was most effective in delaying collagen membrane degradation in clostridial collagenase which is in accordance with the study by Ofer Moses[54] which proved that collagen membranes immersed in 50 mg/ml tetracycline hydrochloride solution exhibited the longest degradation time, both in clostridial collagenase and human bone lineage cell assays in vitro.

In doxycycline, 20mg/ml concentration was found to be more effective in delaying collagen membrane degradation as the new protein release into the medium was less up to day 14 as compared with the group without collagenase both quantitatively and qualitatively.

Quantitative analysis also showed that 100 mg/ml concentration of tetracycline found to exhibit higher new protein release into the medium after day 7 up to day 14. The

reason for the above is low pH which was created by this concentration led to faster collagen degradation compared to group without collagenase.

It is also evident from this study that the semisynthetic tetracyclines, doxycycline are more effective inhibitors of collagenase than the parent compound, tetracycline HCl. This concides with the clinical study which suggested that the administration of doxycycline to humans with periodontal disease appeared to inhibit collagenase enzyme in the periodontal pocket for a longer period of time than tetracycline[9]. The greater inhibitory activity of doxycycline is due to its ability to bind Zn2+ more tightly than the other tetracyclines.

On comparing the mean values of free protein content of group B I with group B II, the results of the present study shows that statistically significant difference ($P < 0.01$) were found at each concentration of tetracycline over day 2, 4, 7 & 14 except in 20 mg/ml concentration, statistically significant difference ($P < 0.0001$) was found only on day 14.

Qualitative analysis using light optical microscope for group B I shows that disorganization and destruction of collagen fibers at tetracycline concentration of 5 mg/ml & 20 mg/ml were first observed on day 2 which steadily increases up to day 14 while disorganization of collagen fibers at tetracycline concentration 50 mg/ml & 100 mg/ml were first observed on day 7 whereas maximum disorganization of collagen fibers at 100 mg/ml was observed on day 14 as compared to 50 mg/ml. So in the present study quantitative and qualitative proved that tetracycline is more effective at 50 mg/ml concentration which is in agreement with Ofer Moses[57] study which proved that collagen membranes immersed in 50 mg/ml tetracycline hydrochloride solution

exhibited the longest degradation time, both in clostridial collagenase and human bone lineage cell assays in vitro.

On comparing the mean percentage of group C I with group C II, the results of the present study shows that statistically significant difference ($P < 0.05$) were found at 5 mg/ml & 20 mg/ml concentration of doxycycline over day 2, 4, 7 & 14 while 50 mg/ml & 100 mg/ml concentration where all the values were not significant. This might suggest that doxycycline 5 mg/ml and 20 mg/ml are more effective in prolonging the collagen membrane degradation time by effectively inhibiting clostridial collagenase.

Qualitative analysis for group C I shows that disorganization and destruction of collagen fibers for doxycycline were first observed on day 4 for 50 & 100 mg/ml concentration which steadily increases up to day 14 while disorganization of collagen fibers at doxycycline concentration 5 mg/ml were first observed on day 7 whereas at 20 mg/ml, it was first observed on day 14. So 20 mg/ml was found to be most effective in delaying collagen membrane degradation qualitatively also. So in the present study quantitative and qualitative analysis led to conclusion that doxycycline is more effective at 20 mg/ml concentration.

So combining quantititave and qualitative results, 50 mg/ml concentration of tetracycline and 20 mg/ml concentration of doxycycline were found to be most effective in delaying collagen degradation in vitro compared to their respective groups without collagenase.

On comparing the most effective concentration of tetracycline hydrochloride with most effective concentration of doxycycline hyclate, statistically significant difference was found on day 2 ($P< 0.0001$) and day 14 which showed doxycyline was more

effective in inhibiting clostridial collagenase enzyme than tetracycline group which is in line with the clinical study which suggested that the administration of doxycycline to humans with periodontal disease appeared to inhibit collagenase enzyme in the periodontal pocket for a longer period of time than tetracycline. So another significant finding of this study was that doxycycline was found to be more effective in delaying collagen membrane degradation than tetracycline when treated with clostridial collagenase in vitro.

Since tetracycline and doxycyline have anti-inflammatory and anticollagenolytic action when immersed in barrier membrane it enhances the structural integrity of membrane thereby increasing the regenerative potential, further research is needed to observe the effects.

SUMMARY AND CONCLUSION

In the present study, our aim was to evaluate the effects of varying concentration of tetracycline and doxycycline on the rate of degradation of bioresorbable collagen membranes by clostridial collagenase when immersed in vitro using spectrophotometric and microscopic analysis.

The present study showed that:

- Both tetracycline and doxycycline modulates the degradation of collagen membranes differently at varying concentrations.
- Free protein release in the medium was less at higher concentration of tetracycline hydrochloride while it was less at lower concentration of doxycycline.
- Tetracycline at 50 mg/ml concentration was most effective in delaying collagen membrane degradation in clostridial collagenase
- Doxycycline at 20mg/ml concentration was found to be most effective in delaying collagen membrane degradation in clostridial collagenase both quantitatively and qualitatively.
- Doxycycline is more effective in delaying collagen membrane degradation in clostridial collagenase than tetracycline or when no drug is present.

This confirms that tetracycline at 50 mg/ml concentration and doxycycline at 20 mg/ml concentration are effective in delaying collagen membrane degradation in clostridial collagenase in vitro. Moreover further research is needed to see the effects of doxycycline on rate of collagen membrane degradation in vivo.

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